Presence of *RHD* in serologically D–, C/E+ individuals: a European multicenter study

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BACKGROUND: *RHD* blood group alleles with reduced or absent antigen expression are a clinically significant and heterogeneous group.

STUDY DESIGN AND METHODS: To detail population genetics data on apparently D– individuals in central Europe, a six-center study was performed with participants from Austria, Germany, Slovenia, Switzerland, and Russia. A total of 1700 serologically D– samples, positive for C and/or E, were investigated.

RESULTS: Observed unexpressed *RHD* alleles were 59 RHD-CE-D+ hybrid alleles, 9 apparently regular RHD, 1 new RHD(Y401X); DELs were 8 RHD(M295I), 6 RHD(IVS3+1G>A), and 1 new RHD(X418L); and weakly expressed RHDs were 2 weak D type 5, 1 weak D type 1, 1 RHD category VI type 1, and 1 novel weak D type 26. Although weak D type 26 was shown to have one of the lowest D antigen densities ever observed, it gave rise to anti-D immunization in a transfused D- individual. **CONCLUSION:** The relative occurrence of *RHD* among serologically D- samples, positive for C and/or E, differed significantly in the investigated central European regions. Considering the growing use of molecular typing techniques, correct identification of blood group alleles with scarce or missing antigen expression is of utmost clinical importance and requires reliable population-based frequency data.

egativity for certain blood group antigens is a basic characteristic of practically all human blood group systems—including RhD—and can also be depicted as naturally occurring double-knockout individuals. In Caucasian persons, the main reason for D negativity was found to be linked to the absence of one of the two closely related RH genes; its molecular background was described.¹⁻³ Approaching RHD in an approximate way, D positivity and negativity could be considered as clearly defined and investigated. Considering antigens of phenotype frequencies with lower than 1 percent, however, further incremental steps are added to this pure positive-negative scheme, as exemplified by weak D and DEL, showing low and minute amount of D antigens, respectively.

ABBREVIATION: PCR-SSP = polymerase chain reaction with sequence-specific priming.

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Received for publication May 27, 2004; revision received August 24, 2004, and accepted September 13, 2004.

TRANSFUSION 2005;45:527-538.

Weak D were shown to be caused by single-point mutations in most cases, leading to amino acid exchanges in the transmembraneous, or intracellular, parts of the D protein and as a consequence thereof showing a reduced D antigen density.⁴ Very weakly expressed weak D, or D only detectable by adsorption-elution techniquesnamed DELs-were frequently unidentified by routine serologic procedures, and the large group of completely unexpressed RHD alleles only became evident by DNAtyping methods. A variety of barely or completely unexpressed RHD alleles were already reported in 1994, among D- individuals with positivity for at least C. The identified alleles included RHD-CE-D hybrids with large parts of the RHCE gene of Caucasian and African origin, the latter found in D- black individuals who exhibited the Cce^s phenotype complex; and apparently complete *RHD* genes, one of which of oriental origin with very low levels of D antigen detectable only by adsorption-elution.⁵⁻¹⁰ Further investigations confirmed these findings and pointed to high incidences of unexpressed RHD alleles among Dindividuals with positivity for at least either C, or E.11-15 Consequently, more systematic and population-based studies characterizing barely or completely unexpressed RHD alleles were carried out and delivered population specific data.

In black African persons, approximately 3 to 5 percent of the population is D-; the vast majority of them are presenting a homozygous or heterozygous combination out of the three predominant D- "alleles": 1) classical RHDdeleted RHd haplotype, 2) unexpressed $RHD\psi$, and 3) D-*RHD*Ce^{S,7,16-18} What really differentiates the black African population from Caucasian persons in this context is the pronounced difference in proportional "allelic" contribution to D negativity. Whereas (1) contributes up to almost 100 percent to D negativity in Caucasian persons, the above-mentioned alleles seem to contribute 56.45 percent (1), 26.2 percent (2) and 17.3 percent (3) in Mali and apparently 32.2 percent (1), 52.3 percent (2), and 15.5 percent (3) in the black South African population.^{17,18} Weak C expression among D- in black African persons is generally encoded by RHDCe^s and therefore correlates directly with the frequency of this allele in the investigated population.⁷ Haplotype frequency of *RHD*Ce^s may be as high as 4.3 percent as shown for black African persons from Mali.18

In Caucasian persons, approximately 15 percent are D–, and the classical *RHD*-deleted RHd haplotype is practically the only contributor (approximately 100%) to this phenotype. The cumulative population frequencies of 1) unexpressed *RHD* genes, 2) D– *RHD-CE-D* hybrid alleles, and 3) DELs were 1 among 6443, 2018, and 3030, respectively, and hence very low compared to black African and Asian persons.¹⁹ In detail, 754 D– individuals with positivity for at least C or E showed 5 individuals with 4 different unexpressed *RHD* genes, 24 individuals with 9 different

RHD-CE-D hybrid alleles with *RHD-CE(2-9)-D* representing by far the most frequent and alone having been observed 11 times, and 15 individuals with three different DEL alleles: *RHD*(M295I), *RHD*(K409K), and *RHD*(IVS3+1G>A).¹⁹ These alleles are expected to contribute to D negativity at a frequency of 0.22 percent for (1) and (2) together and 0.11 percent for (3).

In Asian persons—as shown by studies performed in Japan, Taiwan, and China—D negativity as detected by routine serologic methods includes a high percentage of DELs at a frequency in between 12.8 and 16.3 percent, whereas truly D–*RHD-CE-D* hybrid alleles are found at a comparably low frequency of in between 0.44 and 0.98 percent only.^{8-10,20,21}

It is noteworthy that apparently different region specific DEL alleles are responsible for their common high prevalence in Asian regions, even located close to each other, as exemplified by the Shenzen DEL lead allele *RHD(K409K)* and the Taiwan DEL lead allele *RHD(delEx9)*.^{10,21,22} This regional specificity is further accentuated by the existence of an unexpressed *RHD(G314V)* allele contributing 13.8 percent to D negativity in Japanese (not including DELs), but its complete lack in the other two Asian populations investigated.^{8-10,20-22}

Taking into account the above-mentioned regional specificities, we aimed at investigating "the central European" allelism of D negativity among samples with positivity for either at least C or E. Participants were from Innsbruck (Austria), Oldenburg (Germany), Ljubljana (Slovenia), Bern (Switzerland), Braunschweig (Germany), and Kirov (Russia) and contributed between 54 and 738 samples, adding up to a total of 1700 samples investigated. Participating laboratories and their geographic locations are given in Fig. 1.

MATERIALS AND METHODS

Sample origin

The sample origin was defined by the participating laboratories, which are representatives of the regions investigated: Innsbruck for the federal state of Tyrol (Austria); Oldenburg for the federal states of Lower Saxony, Saxony-Anhalt, Thuringia, Oldenburg, and Bremen (Germany); Ljubljana for different towns of Slovenia, which can be considered as a representative Slovenian population; a vast majority (>80%) of the Swiss samples from Bern from the canton of Bern (Switzerland); Kirov for the regional subdivision Kirov Oblast (Russia); and Braunschweig for the city of Braunschweig and eastern parts of Lower Saxony (Germany).

Serologic typing

For all samples, typing for D, C/c, and E/e antigens was performed by gel matrix testing (Micro ID typing system,



Fig. 1. Participating laboratories and their geographic location in central Europe. Innsbruck, Austria (IBK); Oldenburg, Germany (OLD); Ljubljana, Slovenia (LJU); Bern, Switzerland (BER); Kirov, Russia (KIR); and Braunschweig, Germany (BRA). City of Kirov, Russia, is located outside the shown map.

DiaMed, Cressier sur Morat, Switzerland), including the detection of weak D antigens by indirect antiglobulin test (IAT), despite Oldenburg, which employed an automated blood grouper (PK7200, Olympus, Hamburg, Germany) with a monoclonal IgM (Diagast, Aachen, Germany) and polyclonal IgG (Meridian, Cincinnati, OH), with subsequent IAT for partial and weak D including both tube and gel matrix testing. An estimated 15 percent of the samples from Innsbruck relied on historical data, when testing for weak D was still performed as IAT in tubes. Barely expressed D antigens were confirmed by adsorption of

human polyclonal anti-D from an anti-D-immunized individual with test and control red blood cells (RBC) and subsequent acid elution (Elu-*Kit*II, Gamma, Houston, TX) for further antibody specification.

DNA screening

Ethylenediaminetetraacetate-anticoagulated blood samples were collected for DNA preparation. DNA preparation was performed with a DNA preparation kit (Nucleon BACC2, Amersham Biosciences, Freiburg, Germany) for all samples except Oldenburg, which used another kit (Puregene DNA blood kit, Gentra Systems, Minneapolis, MN).

All samples were screened for *RHD*-specific DNA sequences in the 5'-untranslated region, exon 3 and exon 10 by polymerase chain reaction with sequence-specific priming (PCR-SSP). Screening for exon 3 and exon 10 was performed as described earlier, screening for the 5'-untranslated region was performed exactly as described with primers given in Table 1A (primer 1).¹⁴

The exon scanning method and testing for weak D types 1 to 5 was performed as described earlier, supplemented by the eight PCR-SSPs as described in the next paragraph for additional information on the *RHD* 5'-untranslated region, exons 2 and 8.^{14,23} *RHD* zygosity was determined by checking for the presence of hybrid, upstream, and downstream Rhesus box to predict the presence of a *D*– Rhesus haplotype, by *Pst*I RFLP as described elsewhere.³

RHD DNA sequencing

Genomic DNA of the respective samples was used for specific amplification of RHD exons 1 to 10 and their flanking intronic sequences. All eight reactions worked at the same cycling conditions, which were an initial denaturation step at 94°C for 2 minutes; 10 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 90 seconds, and synthesis at 72°C for 90 s; and 25 cycles of denaturation at 95°C for 15 seconds, annealing at 61°C for 1 minutes, and synthesis at 72°C for 2 minutes. Amplification was carried out in a final volume of 75 µL containing 50 mmol per L KCl, 10 mmol per L Tris-HCl (pH 8.3), 0.01 percent gelatin (vol/wt), 5.0 percent glycerol (vol/wt), 100 µg per mL cresol red, 200 µmol per L of each dNTP, and 250 ng of genomic DNA. Primers are given in Table 1 (Table 1A, primers 1-8 for amplification; Table 1B for sequencing). PCR products were purified with a PCR purification kit (QIAquick, Qiagen, Hilden, Germany). DNA sequencing was performed at the Microsynth DNA service facility (Microsynth, Balgach, Switzerland) with dye terminator technology from Applied Biosystems (Vienna, Austria). The minimal length of the exon-flanking intronic sequences analyzed was 35 bp. New RHD alleles were deposited at the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Alternatively, samples of Oldenburg were sequenced with the dye terminator cycler sequencing method (ABI310, Applied Biosystems, Darmstadt, Germany) as published previously.4,19,24

Enhanced RHD-CE(2-9)-D molecular analysis

The analysis of *RHD-CE(2-9)-D* hybrid alleles was carried out by one PCR-SSP procedure, which detected an *RHD*specific polymorphism located 1038 bp 5' of the intron 1– exon 2 boundary (Table 1C, primer 1); the control primers used and cycling conditions were exactly as described previously.14 PCR fragments for sequencing 5' and 3' breakpoint of RHD-CE(2-9)-D were amplified with reactions D1 (Table 1D, primer 1; exactly as described for C1, without control primers) and long-range PCRs D2 and D3 (Table 1D, primers 2 and 3). Long-range PCR reagents were those from a PCR system (Expand Long Template PCR system, Roche, Vienna, Austria) and exactly used as described by the manufacturer. Cycling conditions were an initial denaturation step at 92° for 2 minutes; 10 cycles of denaturation at 92°C for 10 seconds, annealing at 65°C for 30 seconds, and synthesis at 68°C for 8 minutes; and 25 cycles of denaturation at 92°C for 10 seconds, annealing at 65°C for 30 seconds, and synthesis at 68°C for 8 minutes plus an incremental 20 seconds per cycle; and final elongation for 7 minutes at 68°C. Amplification was carried out in a final volume of 50 µL; PCR fragments were purified and sequenced with primers given in Table 1E as described above.

Detection of D antigen density of weak D type 26

D antigen density of weak D type 26 and control RBCs was determined by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as described.²⁵ Fifty microliters of 2 percent suspensions of washed RBCs in phosphate-buffered saline were incubated at 37°C for 30 minutes with 50 µL of human anti-D. Nine human IgG anti-D monoclonal antibodies (MoAbs) were used: P3x290, P3x35, P3x241, P3x249, and HM16 (Diagast); MS26 and ESD1 (DiaMed); and BRAD-3 and BRAD-5 (provided by G.L. Daniels, Bristol Institute for Transfusion Sciences, Bristol, UK). The human clone AEVZ5.3 served as a negative control. After incubation with the primary antibody, the cells were washed and the bound antibody was detected by the addition of fluorescein isothiocyanateconjugated F(ab')₂ fragment rabbit anti-human IgG (Dako, Glostrup, Denmark). After incubation with the secondary antibody (30 min at 4°C) and washing, 30,000 events per sample were acquired. Absolute D antigen densities (D antigens per RBC) were assessed by use of a standard RBC sample with 18,332 D antigens per cell (provided by V. Curin Serbec and M. Urbajs, Blood Transfusion Center of Slovenia, Ljubljana, Slovenia) deduced from the standard of the "Fourth International Workshop on Monoclonal Antibodies against Human Red Blood Cells." For antigen density calculations, the recommended algorithm was applied.²⁶

Flow cytometric assessment of RhAG expression of weak D type 26 samples and control RBCs was performed by use of murine anti-human MoAb 2D10 (provided by L. de Jong, Sanquin, Amsterdam, the Netherlands). Mouse IgG-negative controls and phycoerythrin-conjugated rabbit anti-mouse IgG $F(ab')_2$ were from Dako.

Image: Construction of the second s	Α	Primer Name	Exon sequencing PCR primer	Gene location	Primer conc.	Product
1 HHDpro-132F GTTAACTCCATAGAGAGGCAGCAGAA Exon 1 350 2 nHHD_1-1405F CATTTACCCATATAGAGAGAAGAGAACA Exon 2 350 - 2 nHHD_2+01R GGCAATATCCCAAGATCTTCTGGAACC Exon 2 350 - 3 RHD_2+181F GGCACTTCTACGGGAGAGAAGA Exon 3 400 3 RHD_3+36F AGGCACTTCACCACCATCCTA Exons 4 and 5 300 3 RHD_1-3+57R GCACTGCACAGCATCCAGACTCCAGACTCC Exons 4 and 5 300 4 nRHD_1-5+57R GGGAGGCACCAGCGACCAGACAGA Exon 7 350 5 RHD_1-7-325F TGGGAGGCACCTCCACAGCAGACAGA Exon 8 350 6 nRHD_1-7-325F TGGGAGGCACCCCCAGAGCAGAC Exon 8 350 7 RHD_8+57F TGGAGGCACTCCACACATGCACACAGACACC Exon 10 350 8 RHB1-17F TGGAGGCACACCCCCAGCAGCAGCACA Exon 10 350 8 RHB1-17F TGGAGGCACACTCCACATTGCACACATACTTC Exon 9 350 7 RHD_8+57F TGGAGGCACCCCCAGGGAGACACA Exon 10 <td>1</td> <td></td> <td></td> <td>Evon 1</td> <td>350</td> <td></td>	1			Evon 1	350	
2 nHHD_11-tagsp CATTCCCCTATTAACAGACAAGA Exon 2 350 2 RHD_12-182F AGGCATCCCAATCTGGAACC Exon 3 400 3 RHD_13-35F AGGCATCCTAACGGGAGAAGAG Exon 3 400 3 RHD_13-36F AGGCATCTCAGGGCTCCCCCCCTCC Exon 3 400 3 RHD_13-45F AGGCAGTGACCCCCCCCTCCC Exon 4 400 4 nPHD_15-1483F AGGCAGTGGCCCCCTCACC Exon 5 300 4 nPHD_15-1483F AGGCAGTGGCCCCCCCACC Exon 7 330 5 RHD_17-322F TGGAGGCTCTGAAGCACAGCAGCAGCGCCCCAAGCAAAG Exon 7 350 6 RHD_18-151R GCTCTCAATCTGAAGTCCCACAGCACAACTCTC Exon 8 350 7 RHD_18-67F TGAGAGTCTGCGGGCACACCACACACATCTC Exon 9 350 7 RHD_18-417F GCAGTCGAGCACCTGCACACACATCTC Exon 9 350 8 DEX105P-135B-B CAGTCCACCGAGCACTGCACACACATCT Exon 10 350 7 RHD_18-417F GCAGTCCACCCCACCCCCACCACATCT Exon 10 350	1	BHDpro-132E	GTTAACTCCATAGAGAGGGCCAGCAGAA	Exon 1	350	302
2 PHO_2+81F GGCATATCCCCAGATCTCTGGAAGC Exon 2 350 3 RHD_3+30F GGCATCCTTACGGGAGAAGAG Exon 3 400 3 RHD_3-30F GGCATCCTTACGGGAGAAGAGG Exon 3 400 3 RHD_3-45F AGGCATCTACGGGGTGCCCGTCA Exons 4 and 5 300 4 nRHD_15-148P CCACTGTACCACCCCGCCTCA Exons 6 350 4 nRHD_15-148P CCACTGCACAGTGCCCATCAGTCCC Exon 6 350 4 nRHD_16-160F CTCTTGTATTCACCACCCCCCCCACAG Exon 7 350 5 RHD_17-327F TGGAAGCTCCACATTGCAGATACCCCCATA Exon 8 350 6 nRHD_18-161F CAGATCCCACATAGCACACATACTCC Exon 9 350 7 RHD_18-417F TGAGATCCACATATAGCCCACACATACTCC Exon 10 350 8 RHSH=417F CAGTCCCACGTGCACACATAGCCACACATACT Exon 10 350 8 RHSH=417F CAGTCCCACGTGCACACCC Exon 10 350 7 RHD_18-427F TGAGATCCACATTAGAGCC Exon 10 350 8	2	nRHD i1-1405F	CATTTCCCCTATTTAACAGACAAGAACAAG	Exon 2	350	1710
3 RHD_2+3201R AGGCCACCTTAACGGGGAGAAGAG Exon 3 400 3 RHD_3-3501R AGGACTATCAGGGCTCCA Exon 3 400 3 RHD_3-45F AAGGACTATCAGGGCTTCCCCCGTCC Exon 4 and 5 300 4 nRHD_15-1487 AGGCAGTAGCACCCACGCACTCCA Exon 6 350 4 nRHD_16-1480F AGGCAGTAGCACGCACGCACACAGTCCA Exon 7 350 5 RHD_16-169F CTCTTCATTCAACACACACACACACACACATTCCCCGA Exon 7 350 6 RHD_18-11R TGGGACACACTCCACACATTCGACACACACATTCC Exon 9 350 7 RHD_18-11R CACATCCACACACACACACACATTCCCCGA Exon 10 350 8 DEX10-SP-1328R GTTTTACTCATAACAGCACACACACATTCC Exon 10 350 8 DEX10-SP-1338- CACATCCACACCCCCC Exon 10 350 7 RHD_18-417F ATTCCAGTCAACTTGACCCCC Exon 10 350 8 DEX10-SP-1338- CACATCCAACATTGAGCCC Exon 2 SY14-103R TCAAGCACACATGACACCACCCC Exon 10 5Y18-130F GTCACACATCAACTCAACATCTGACCCCC	2	RHD i2+61R	GGCAATATCCCAGATCTTCTGGAACC	Exon 2	350	
3 RHD_I3-301F GCTATGTTGCCAGCTCGGTCC Exon 3 400 3 nHD_I3-45F AGGACTACGGGTGCCCCCCGC Exons 4 and 5 300 4 nRHD_I5-1489R CCACTGTGACGACCCCCCCCA Exons 4 and 5 300 4 nRHD_I5-17R GCACTGCACAGTGCACCTCAGGTCC Exon 6 350 5 RHD_I5-160F CTCTTCATTTACACAACTCCCCCCAC Exon 7 330 6 nRHD_I5-160F TGGTGCACAGTCCACGTGCAGGAG Exon 7 350 6 nRHD_I8-17R TGGAGGCTGTCCACAGTCGAGGAGA Exon 8 350 7 RHD_I8-187F TGGAGGCCTTGCACAGTCGAGGAGA Exon 10 350 8 RHI8-417F CACATCGCCTGAGCCAAGACGAACATC Exon 10 350 8 RHI8-417F CACAGCCGCTGCAGCACAGGGCAACATG Exon 10 350 8 SYpro-118F CACAGCCAGCCTGCAGCCAAGGGAACC Exon 10 350 8 SYH-102R CACAGCAGCCTGCAGTCGAGGGAGG Exon 10 350 9 SYP-118F CACAGCAGCCTGCAGTGAACCTGCAGGGAGG Exon 10 550	3	RHD_i2-182F	AGGCCACCTTAACGGGAGAAGAG	Exon 3	400	677
3 RHD_B-48FF AGGACTATCAGGGCTTGCCCGTGC Exons 4 and 5 300 3 RHD_B-14987 AGGCAGTAGCAACGTGGCCCTCA Exons 6 350 4 nRHD_B-16-17 GCACTGACCACTGAGCACTCAGGTCCC Exon 6 350 5 RHD_D-6-160F CTCTTCATTTCAACAACTCCCCCACACGACCA Exon 7 350 6 RHD_D-2-32FF TGGAGGCACGTCCAACGCACACACACACCCCCA Exon 8 350 7 RHD_D-8-161F GCCTCAACAGTCCACTACACACATCTC Exon 8 350 7 RHD_D-8-162R TTTTACTGAGGCACACGCACACACACATCT Exon 8 350 8 RHH-9-17-32FF TGGAGGCACAGCACAGGCACACCACACATCT Exon 9 350 8 RHH-9-17-32F TGGAGGCACACGCC Exon 10 350 8 RHH-9-17-32F CACTGCCACCTGAGCGACACAGCACACAC Exon 10 350 8 RHH-9-17-32F CACTGCCACCTGAGCGAGCACC Exon 1 350 9 Primer Name Exon 3 350 50 9 SYIP-118F CACTCCACCTGACCTGAGCAGCACACACACACC Exon 1 50	3	RHD_i3+301R	GCTATGTTGCCCAGCTCGGTCC	Exon 3	400	
3 RHD_I5-149R 4 CCACTGTGACCACCCAGCATCA Box no 6 Ston 6 Ston 350 4 nRHD_I6-157R 6 GCACTGCACAGTGGCCCTCA CACAGTCCCCCACA Box no 7 Ston 350 5 RHD_I7-327F 7 TGGAGCACGTCCACAGCACAG CCCCACAGCACAGTCCCCCACA Box no 7 Ston 350 6 RHD_I7-327F 7 TGGAGCACGTCCACAGCACAG CCCACAGTGCCACAGCAG Box no 8 Ston 350 6 RHD_I7-327F 7 TGGAGCACGTCCACAGCACAGTGCCACAGCAG Box no 8 Ston 350 7 RHD_I8-151R 7 CGACTCCACAGCCTGACAGTG GCCCCCCCCCACACAGTG CCCCCCCCCC	3	nRHD_i3-45F	AAGGACTATCAGGGCTTGCCCCGTGC	Exons 4 and 5	300	983
4 nRHD_I6+1463F AGGCAGTAGCCAGTGGCCCTCA Exon 6 350 4 nRHD_I6+160F CTCTTCATTTCAACAACTCCCCCATAGGTCC Exon 7 350 5 RHD_I7-32FF TGGAGGCACGCCCACAGGCCCCACAAG Exon 7 350 6 nRHD_I7-32FF TGGAGGCCTCTGAGAGCTCCCACAATACTCCC Exon 8 350 7 RHD_I8+7FF TGGAGGCCTCACAGGCCCACAATACTCCC Exon 9 350 7 RHD_I8+7FF TGGAGGCCTGACAGCCC Exon 9 350 8 RH8+117F CATCCAGGCCTGACAGCCC Exon 10 350 8 RH8+117F CATCCAGCCTGAGCAGCCC Exon 10 350 8 SY1+417F ATTCACTTGAGACACCGC Exon 10 350 9 SY1+417F ATTGCAGCCCCCCTGTGAGCC Exon 10 350 9 SY1+4103R TGGAGCACCCCCCTGTGAGAGCC Exon 5 SY1+4103R TGGCACACCCCAGCTTGAGAGCC Exon 10 9 SY1+4103R TGGCACACCCCAGGGGAGAGCA Exon 10 400 (mmolL) [em 19 SY1+4105R TGCACAGCCAGGGAGAGCAC	3	RHD_i5+149R	CCACTGTGACCACCCAGCATCCTA	Exons 4 and 5	300	
4 nRHD_leis7R GCACTGCACAGTGGCCCTCACAGTCC Exon 6 350 5 RHD_17-327 TGGCACCACGTCCACAGCACAG Exon 7 350 6 RHD_17-327 TGGCACCACGTCCACAGCACAG Exon 8 350 6 RHD_18-151R GCCTCACAGTCCACAGTCCACAGCAG Exon 8 350 7 RHD_18-151R GCCTCACAGTCCACAGTCACAGCAG Exon 9 350 7 RHD_18-157R GCACTCCAGCGCAACAGTC Exon 9 350 8 RH9-17F GCACTCCAGCGCGAACAGTC Exon 9 350 8 RH9-17F GCACTCCAGCCTGAGCAGTC Exon 10 350 8 RH9-17F GCACTCCAGCCTGAGCAGC Exon 10 350 9 SY1-13F GCACGCCAGCCTGAGCAGC Exon 10 350 9 SY1-147F GCACAGCCAGCCAGTGAGCAGC Exon 6 SY1-147F 9 SY1-147F GCACAGCCCAGGTGAGCAGC Exon 6 SY1-147F 9 SY1-147F GCGCAGCCCAGGTGAGCAGCAGCAGC Exon 6 SY1-147F 9 SY1-147F GCGCGCACGCA	4	nRHD_i5+1463F	AGGCAGTAGCGAGCTGGCCCCTCA	Exon 6	350	554
5 RHD_I6+160F CTCTTCATTCAACAAACTCCCCGA Exon 7 350 5 RHD_J7-322F TGGAGACCGTCCACAGCAACAACACAACAACAACTCC Exon 8 350 6 nRHD_B+05F TGGAGACCTCTAGAAGACTTGCCGG Exon 8 350 7 RHD_B+05F TGGAGACCAGTCCACAACACACACACACACACACACACAC	4	nRHD_i6+57R	GCACTGCACAGTGGCCCATCAGGTCC	Exon 6	350	
5 RHD_J7.328/R TGGGAGCTCGACAGCTAGCAAGA Exon 7 350 6 RHD_JR-327 TGGGAGCTTGACAGTGTGCGGG Exon 8 350 7 RHD_J8-67F TGGAGCTTGACAGTTGGCGG Exon 9 350 7 RHD_J8-67F TGGAGACCTGCACATTAGCACATTG Exon 9 350 8 DEX10-SP-138-as CACTGCCACCACATTG Exon 10 350 8 DEX10-SP-138-as CACTGCCACCTGCACGACACATG Exon 10 350 8 DEX10-SP-138-as CACTGCCACCTGCACGACACACTG Exon 10 350 8 DEX10-SP-138-as CACTGCCACCTGCACGACACCT Exon 10 350 8 TGGAGACTTGAAGACACCACTG Exon 10 350 S0 8 TGGAGACTGCAACATTGAAGACCACCACC Exon 1 S0 S0 S0 9 SVG-13F TGGACACTTCAACATTGAAGACCACC Exon 4 S0 S0 9 SVG-13F TGGACACTTCAACATTAGACGCACC Exon 5 S0 S0 9 SVG-147F CCTAGGCACGACAGACACCACGTAG Exon 10 Concorcorcorcorcorcorco	5	RHD_i6-160F	CTCTTCATTTCAACAAACTCCCCGA	Exon 7	350	665
6 NHD_17:32/F IGAAGACH IGAAAACH IGACGG Exon 8 350 7 NHD_18-67F TGAGATACTGTCGTTTTGACAACAATACTTC Exon 9 350 7 NHD_18-67F TGAGATACTGTCGACAACAACAACAATACTCC Exon 9 350 8 PHMEY17F CAATGCACGCGAAACAACAACAACAACAACAACAACAACAACAA	5	RHD_i7+326R	TGGGAGCACGTCCACAGCAAAG	Exon 7	350	
b NHHD_B+07F TGAGATACTTGGTTTGACACAATACTTC Exon 9 350 7 RHD_9+02R GATTTACCATAAACAGCAATACTTC Exon 9 350 8 DEXIOSP-1358-gs CATTGCCTGCCCCAACATACTCC Exon 10 350 8 DEXIOSP-1358-gs CATTGCCTGCCCAACACATG Exon 10 350 8 DEXIOSP-1358-gs CATTGCCTGCCCAACACATG Exon 10 350 8 DEXIOSP-1358-gs CATTGCCTGCCCAACACTGCACCAACC Exon 10 350 8 DEXIOSP-1358-gs CATTGCCTGCCCAACACATGCAACC Exon 1 350 9 SYM-119F CACAGCCACCCTTGCAGCACC Exon 2 SYX-151F GACATGCAACCATGAACACC Exon 3 9 SYM-13F GCACAGCCACCCACCTGAGGC Exon 6 SYM-121F GCTGCAACATTCAAGCACCGTGG Exon 7 9 SYM-13F GCACAACCTCCACCTGCAGGAGGC Exon 8 SYM-13F GCACAACCTCCACCTCGAGGACGAC Exon 10 9 SYM-13F GCACAACTTCAACATATAACCCCAGG Exon 10 GC Primer Name Intron 1 RHD-SSP Gene locatin (mmol/L) len	6	RHD_1/-32/F	IGGAGGCICIGAGAGGIIGCGG	Exon 8	350	603
1 HHD_B+6/F IGAGARAC IG ICGT IT GAGARACAGAAATTIC Exon 9 350 7 HHD_B+62R GTTTRCOATAACAGCAACAGAACTATATCC Exon 10 350 8 RHI=417F CAGTGCCTGGGGAAACATG Exon 10 350 8 RHI=417F CAGTGCCTGGGCGAACATG Exon 10 350 8 RHI=417F CAGTGCCAGCCTGGACAGAGCTGCAGAAC Exon 10 350 8 YT STACAGCCAGCCTGGACAGACGTGCAGCGAAC Exon 10 350 8 YT TAGTGAGCAGCGCGAGCGGAGGAGGCGAACACTGAGAGAGA	6	NRHD_18+151R	GCCTCACAGTCCACATTAGCAGCAG	Exon 8	350	000
7 PHID_9H02H EXTINCE-138-as CARTOCCTGGCGCAACATTG Exon 10 350 8 Primer Name Exon 20 Stop 350 9 Primer Name Exon 20 Stop 350 9 Primer Name Exon sequencing, sequencing primer Gene location 9 SY10-119F CACAGCCAGCCTTGCAGCC Exon 1 350 9 SY11-147F ATTCAGTTGAGAGCC Exon 3 SY11-147F 9 SY10-149F CCTAGAGCACCTCAGTGAGGC Exon 3 SY11-147F 9 SY10-149F TCACTGAGGCACCTCAGCTGAGGC Exon 5 SY15-149F TCCACACTGAGGCTGAACACCTGAGGC Exon 5 9 SY10-149F TCCACACTGAGGCACAGGAGGC Exon 10 SY16-149F TCCAACATTGAGTCAGCCAGGGAGGC Exon 10 9 SY19-119F TCCAAGATCAACTTGAGAGCCAGGTAG Exon 10 Immol 1 400	1	RHD_18-67F		Exon 9	350	268
b DEAT0-57-1358-38 CARTECLAGECGAACAAGCAGAACAACAACAACAACAACAACAACAACAA	/	RHD_19+62R	GITTIACICAIAAACAGCAAGTCAACAIAIAICCI	Exon 9	350	F07
a Primer Name Exon concentration Exon 1 B Primer Name Exon sequencing, sequencing primer Gene location SYpro-118F CACAGCCAGCCTTGCAGCC Exon 1 SY1-147F ATTCAGTTGAGACACATTGAGGC Exon 2 SY1-147F CATAGGACACCTGTGAGAGC Exon 3 SY1-147F CATAGGACACCTGTGAGAGC Exon 3 SY1-147F CATAGGACACCTGAGAGACACC Exon 5 SY1-148F TCCAGAGCTCACTGAGAGAC Exon 5 SY1-148F TCCAGAGCTCACTGAGAGACGC Exon 6 SY16-149F GTGCACCACACTGAGGACAGGGA Exon 7 SY17-121F ATGTACCAGCCACGGAGAGAGGAC Exon 10 C Primer Name Intron 1 RHD-SSP Gene location RHD-11-042F GGGTGACAGTCATTCTCGAT Intron 1 400 1 RHD-11-042F GGGTGACACTTACTCAGAGACAAGGTC Intron 1 400 1 RHD-11-042F GGGTGACCACTCCTCATTTACAGAACAGACAAGG Intron 1 400 2 RHD-11-042F GGGTGACCACTCCTCAGGTTGCA Intron 1 400 1 RHD-11-042F GGGTGACCACGTCCTCAGGTTGCA Intron 1 400 2 RHD-11-1403F CATTTCCCCTATTTACAGACAGAACTGTGGTG Intron 1 400 3 RHD-11-1403F GACAAACT	8	DEX10-SP-1358-as		Exon 10	350	567
B Primer Name Exon sequencing, sequencing primer Gene location SYNI-147F ATTCAGTTGAGACC Exon 1 SYNI-147F ATTCAGTTGAGACCC Exon 2 SYNI-147F GATGGAACGCCACCTTGAGAGC Exon 3 SYNI-147F CCTAGAGCTCACACTCTAGAGGC Exon 4 SYNI-149F TCCACTGATGAAGGCCACTCTAGAGGC Exon 6 SYNI-149F TCCACTGATGAAGGACCACGCAG Exon 6 SYNI-121F ATGTACCAGCCAGGGGAGAGG Exon 7 SYNI-121F ATGTACCAGCCAGGGGAGAGG Exon 10 C Primer Name Intron 1 RHD-SSP Gene location RHD-11-1042F GGGTGACGAGTGAAACTCTGTCCAAT Intron 1 400 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGC Intron 1 400 1 RHD-11-783F GACAAACTTGGGTCAAAACAGGAGCACAAGA Intron 1 400 2 RHD-11-783F GACAAACTTGGGTCAAATCAGGAGGACT Intron 1 400 2 RHD-11-783F GACAAACCTTGGCTCAATCAGGAGGC Intron 1 400 2 RHD-11-783F GACAAACCTGGGTGCAACAGGAGGC Intron 1	0	Drimer Neme			350	
STIDO-118F CACAGCCARCCATGAGCU Exon 1 SY11-147F ATTCAGTTGAGAACATTGAGG Exon 2 SY12-151F GAGATGGTCACTCCACTCAGAGC Exon 3 SY14-103R TGATGGAAGGGCTCCACTGTAGAGGC Exon 4 SY18-127R CCTAGAGCTCCACTGTAGAGGC Exon 5 SY18-139F GTGCACATTCAAGTCTAGAGAG Exon 7 SY19-51P TCCAAGACCACTATATACCCAGG Exon 7 SY19-51P TCCAAGACTCTACCACCAGG Exon 9 SY19-51P TCCAAGATCATCAGCAGGAGAGAC Exon 9 SY19-51P TCCAAGATCATCAGCACAGT Intron 1 400 RHD-11-1042F GGGTGACGAGTGAAACTCTGCGAT Intron 1 400 RHD-11-1042F GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 1 RHD-11-1405F CATTCCCCATTTTACAGACAAGAACAAGA Intron 1 400 2 RHD-11-1405F CATTCCCCCATTTACAGGAGTC Intron 1 400 1 1 RHD-14-1405F CATTCCCCCATTTACAGGAGTCG Intron 1 400 1 1 1 1 00 1 2 Ethol-1-1-783F GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 1 00 1<			Exon sequencing, sequencing primer			
SY11-14/F AI ICAGI ISAGAAAATI GAGGC Exon 2 SY12-151F GAGGGGTCACCACTCATGAGGC Exon 3 SY18-103R CGTGGAAGGGCTCACCACTGTAGG Exon 4 SY18-149F TCCACTGATGAAGGCCCACGTAG Exon 6 SY17-121F ATGTACCACACTCAAGAGGC Exon 7 SY17-121F ATGTACCAACCCAGGGAGAGGAC Exon 8 SY19-119F TCCAAGATCTACCATTATATACCCAGG Exon 10 Primer Name Intron 1 RHD-SSP Gene location (mmolL) lene RHD-11-1042F GGGTGACCAGTGAAACTCTATCTGGAT Intron 1 400 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGGAGT Intron 1 400 1 nRHD-11-1405F CAAAACTTGGGTTCAAAACAGGAGGC Intron 1 400 2 Primer Name Breakpoint long-range PCR primer Gene location (mmolL) lene 1 nRHD-11-1405F CAACAACTTGCGTCTACAGAACAAG Intron 1 400 1 2 RHD-11-783R GACAAACTTGCCTCAAGTGCAG Intron 1 400 1 2 RHD-11-783R GACAAACTTGGCTCTCAGAGTGCA Intron 1 300 1 3 DED-10-791585-85 CAGTGACCAACAGGAGGGTCCA Intron 1 300 1 2 RHD-1-7358F CACAACCTGAGGGTGCA </td <td></td> <td>SYpro-118F</td> <td></td> <td>Exon 1</td> <td></td> <td></td>		SYpro-118F		Exon 1		
SY12-151F GACAGGICACICCCCIGIGIAG Exon 3 SY14-103R TGAAGGGCTCCACGTAGACC Exon 4 SY15-149F TCCCACGTGAGAGGCCGTAG Exon 5 SY15-149F TCCCACGTGAGAGGACCGTAG Exon 6 SY16-130F GTGCACATTCAAGTCTGAGAAG Exon 7 SY17-121F ATGTACCAGCCAGGGAGAGGAC Exon 8 SY19-58R CAAGTCAACATATTACCCCAGG Exon 9 SY19-519F TCCAAGATCTCTCCAATTCAG Exon 10 Primer Name Intron 1 RHD-SSP Gene location (nmol/L) leng RHD-11-783R GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 RHD-11-783R GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 1 NRHD_11-1405F CATTTCCCCATTGAAATCAGGAGTC Intron 1 400 1 RHD-11-783R GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 1 RHD-11-783R GACAAACTTGGTTCGAATCTCGGAT Intron 1 400 1 RHD-11-783R GACAAACTTGGTTCGAATCTCGGAT Intron 1 400 1 RHD-11-783R GACAAACTTGGTTCGAATCTCTGGAT Intron 1 exon 3 300 3 RHD-14-88F GACGAACAGCACCTCCTCAGGTTGCC Intron 1 exon 3 300 3 RHD-18-88F GACGACAAGTTCTGTGTGGC Intron 1 exon 10 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-11-738F CACACACAGTTGGTTCAATCCGC Intron 1 RD-11-738F CACACACAGTTGTGTGTGG Intron 1 RD-11-738F CACACACAGAAGAAGAGGG Intron 1 RD-11-738F CACACACAGTTGAGTTGTGTGTG RD-12-2403F GAGTTAATTCCCCC Intron 2 RD-22-403F GAGTTAATTCCCACCGGGAGTGG Intron 1 RD-12-2403F AACTGGCGTGGTGTCA Intron 2 RD-22-403F AACTGCAGTGGTGTTCA Intron 2 RD-22-403F AACTGCAGTGAGGAGTGG INTCA 1 RD-12-2403F AACTGCAGTGTGTGTCA Intron 9 RD19-429F CACACCGTGAGTGTGTCA Intron 9 RD19-429F GAGTGGTGAGCAGTTGTCTGTGGGCAGG Intron 9 RD19-2207R GCCAATGGAGTGTCTCAGGCAG Intron 9 RD19-2207R GCCAATGGAGGAGTGGTCC Intron 9 RD19-2207R GCCAATGGGGGAGGCC Intron 9 RD19-2207R GCCAATGGGGGGCCGGG Intron 9 RD19-2207R GCCAATGGGGGGCCGGG Intron 9 RD19-2216F AGGTGGGAGGAGTTCTGCC Intron 9 RD19-2216F AGGTGGGGAGGCTCTGCCAGG Intron 9 RD19-428F GAAGTGTTCCAGTGCCCG Intron 9 RD19-		SY11-14/F	ALICAGIIGAGAACAIIGAGGC	Exon 2		
STIA-1103H IGANGGC 11CARACGC EXON 5 SYI5-130F CTGAGGCTCCACTGTAGAGGC EXON 5 SYI5-149F TCCACTGTAGAGGCCACGTAG SYI5-130F GTGCCACATTCAGTCTGAGAGG EXON 7 SYI7-121F ATGTACCAGCCAGGGAGGAGGAC EXON 9 SYI9-119F TCCAAGATCTCTCCAATTCAG EXON 9 SYI9-119F TCCAAGATCTCTCCGAT Intron 1 400 RHD-11-783R GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 Primer conc. P Primer name Breakpoint long-range PCR primer Gene location (nmol/L) len 1 nRHD_11-783F GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 2 RHD-11-783F GACAAACTTGGTTCAAATCAGGAGTC Intron 1 400 2 RHD-11-783F GACAAACTTGGTTCAAGACAGGAGCAC Intron 1 400 2 RHD-11-783F GACAACTTGGTTCAACACGAGGGCT Intron 1 400 3 DEX10-5P-133B-as CAGTGCAGTGAAACTCTGTCGGAT Intron 1 400 3 DEX10-5P-133B-as CAGTGCCACTCAGGAGGCACT Intron 1 exon 3 300 3 DEX10-5P-133B-as CAGTGCCACTCAGGGGCC Intron 1 exon 10 300 E Primer Name Intron sequencing primer Gene location RD-11-738F CACAGCAGCAGGAACTTGGC Intron 1 exon 10 300 E Primer Name Intron sequencing primer Gene location RD-11-738F CACAGCAGGAACTAGGAGCG Intron 1 RD-11-738F CACAGCAGGAACTTGGTGG Intron 1 RD-11-738F CACAGCAGCAGGAACTGGCCA Intron 1 RD-11-738F CACAGCAGCAGGAACTGGG Intron 1 RD-12-9495R AACGGGCAACTTGGTGG Intron 1 RD-12-9495R AACGGGCATGTAGGTGGCCA Intron 2 RD-12-9495R AACGGGCATGTAGGTGGG Intron 2 RD-12-9495R AACGGGCATGTAGGTGG Intron 2 RD-12-9495R AACGGGCATGTAGGGTGG Intron 9 RD19-4295F CCAGCCAGGGGGGGGGG Intron 9 RD19-4295F CCAGCCAGTGTTGATCCCC Intron 9 RD19-4295F CCAGCTGATGTAGCTGAGGGG Intron 9 RD19-4295F CCAGCTGATGTAGCTGAGGGGG Intron 9 RD19-4295F GAAGTGATGTCAGGGCGAGG Intron 9 RD19-4295F GAAGTGATGTCAGGGCGAGG Intron 9 RD19-4295F GAAGTGATGTCAGGGCGAGG Intron 9 RD19-4295F GAAGTGATGCATGTCAGGCGCGAG Intron 9 RD19-4295F GAAGTGGTGGGCAGCTCTGGCGGGG Intron 9 RD19-4295F GAAGTGGTGGGCAGCC Intron 9 RD19-4295F GAAGTGGTCCACCGGCGGCC Intron 9 RD19-4295F GAAGTGGTCCACCGGCGGCC Intron 9 RD19-4295F GAAGTGGTCCACCGG		SY12-151F	GAGAIGGTCACTCCACTCTGTAG	Exon 3		
STIB-127H DCIADAGDICAC GIAGAGOC Exon 6 SYI6-130F GTGCACATTCAAGTCTGAGAAG Exon 6 SYI7-121F ATGTACCAGCCAGGGAAGAGAC Exon 8 SYI9-58R CAAGTCAACATATATACCCAGG Exon 9 SYI9-58R CAAGTCAACATATATACCCAGG Exon 9 SYI9-58R CAAGTCAACATATATACCCAGG Exon 10 Primer Name Intron 1 RHD-SSP Gene location RHD-11-1042F GGGTGACGAGTGAACTCTATCTCGAT Intron 1 400 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTGA Intron 1 400 1 nRHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTCA Intron 1 400 2 Primer Name Breakpoint long-range PCR primer Gene location (nmol/L) len 1 nRHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTGC Intron 1 400 1 2 RX3-US-451-as ACTGATGACGAACTAACTATGGGTTCAATCAGGAGTGC Intron 1 400 1 2 RHD-851-as ACTGATGACCACTCATCATGGTGCC Intron 1 400 300 1 2 REX10-SP-1358-as CAGTGACGAACAACTAGAAGAACGAGGA Intron 1 400 300		SY14+103R		Exon 4		
STIB-189T CGGCACATTCAAGTCTGAGAAG Exon 7 SYI7-121F ATGTACCAGCCAGGAGAGAGGAC Exon 8 SYI7-121F ATGTACCAGCCAGGAGAGAGGAC Exon 9 SYI9-58R CAAGTCAACATTATACCCAGG Exon 10 Primer conc. P C Primer Name Intron 1 RHD-SSP Gene location (nmol/L) leng RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGCGAT Intron 1 400 400 MHD-17-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 (nmol/L) leng 1 nRHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 400 (nmol/L) leng 1 nRHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 400 1 2 EXU-9-51-38 GGTGACGAGTGAAACTCTACTCGGAT Intron 1 400 1 300 </td <td></td> <td>SYI5+12/R</td> <td></td> <td>EXON 5</td> <td></td> <td></td>		SYI5+12/R		EXON 5		
SY17-121F ATGTACAGCCAGGAGAGAC Exon 8 SY19-58R CAAGTCAACATATATACCCAGG Exon 9 SY19-58R CAAGTCAACATATATACCCAGG Exon 9 SY19-58R CAAGTCAACATATATACCCAGG Exon 9 SY19-58R CAAGTCACTCTCCAATTCAG Exon 9 SY19-519F TCCAAGATCTCTCCAATTCAG Exon 9 Primer conc. P (mmo/L) lenv RHD-11-738R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 Primer conc. P Primer Name Breakpoint long-range PCR primer Gene location (mmo/L) lenv 1 nRHD_11-1405F CATTCCCCTATTTAACAGACAAGAACAAG Intron 1 400 1 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 2 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 2 RHD-11-1405F CATTCCCCTATTTAACAGACAAGAACAAG Intron 1 400 2 RHD-11-1402F GGGTGACGAGTGAAACTCTGATCTCGAT Intron 1 400 3 DEX10-5P-1358-as CAGTGCACGCGGAACATTG Intron 1 -exon 3 300 3 DEX10-5P-1358-as CAGTGCCTCCCAGGTTGCC Intron 1 -exon 10 300 3 DEX10-5P-1358-as CAGTGCCTGCGGAACATTG Intron 1 -exon 10 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-11-788F CACACAGTGAGGGGAGCGA Intron 1 RD-12-9495R AACCAGCATGCCCC LOCAGGTTGCC Intron 1 RD-12-9495R AACCAGCATGCAGTGGCA Intron 1 RD-12-9495R AACCAGCATGAGGGTGCCA Intron 1 RD-12-9495R AACCAGCATGAGGGTGCCA Intron 1 RD-12-9495R AACCAGCATGAGGGTGCCA Intron 1 RD-12-9495R AACCAGCATGAGGGTGCCA Intron 2 RD-12-9495R AACCAGTTAGCCTGCCCC Intron 2 RD-12-9495R AACCAGTGTGACATCATAGC RD-12-9495R AACCGGCATGGTGGTCA Intron 2 RD-12-9495R AACCGGCAGGGGGGGG Intron 2 RD-12-9495R AACTCCCCCCCGGGAATCT Intron 9 RD19-4795F CCAGCCACGTGAGGTGG Intron 9 RD19-4795F CCAGCCAGTGGTGCACCGGGAGGG Intron 9 RD19-4296F GTTATGCTCGGGGAGCGGG Intron 9 RD19-4296F GATTATGCCTGGTGGTGCAAGGG Intron 9 RD19-2916F AGGTGGGCACAGTTCAGGTGG Intron 9 RD19-2916F AGGTGGGGACCATTGCAGGTGG Intron 9 RD19-915F GGAGTGCATGCTTGCAGGTGGCCA Intron 9 RD19-915F GGAGTGCATGCATGCCAGGTG Intron 9 RD19-915F GGAGTGCATGCATGCCAGGTG Intron 9 RD19-9216F AGGTGGGCACAGTTCCTGG Intron 9 RD19-925F GGAGGGGGATCCTTGCAGGTGGCCAGG Intron 9 RD19-915F GGAGTGCATGCATGTCAGGTGGCCG Intron 9 RD19-945F GGAGTGCATGCATGCCGGGTG Intron 9 RD19-945F GGAGTGCATTCAAGGTTGCCCG Intro		STID-149F SVic 120E		EXON 6		
SY19-58R CAAGTCAACATATATACCCAGG Exon 9 SY19-119F TCCAAGATCTTCTCCAATTCAG Exon 10 C Primer Name Intron 1 RHD-SSP Gene location (mov/L) len RHD-11-1042F GGGTGACGAGTGAAACTCTATCTCGAT Intron 1 400 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 D Primer Name Breakpoint long-range PCR primer Gene location (mov/L) len 1 nRHD-11-783R GACAAACTTGGGTTCAAATCAGGAGAGC Intron 1 400 400 1 1 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGAGC Intron 1 400 400 1 400 1 400 1 400 1 400 1 400 1 400 1 400 1 400 1 400 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 1 400 1 1 1 1 1 1 1 1 1 1 1 1 1 1		SVi7-101E		EXON 8		
Strisht Order Transconder Exon 10 C Primer Name Intron 1 RHD-SSP Gene location (rmm/L) len RHD-11-1042F GGGTGACGAGTGAAACTCTATCTCGAT Intron 1 400 400 Primer Name Breakpoint long-range PCR primer Gene location (rmm/L) len D Primer Name Breakpoint long-range PCR primer Gene location (rmm/L) len 1 nRHD-11-1042F GGGTGACGAGTGAAACTCTATCTCGAT Intron 1 400 400 2 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGGAT Intron 1 400 400 1 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGGAT Intron 1 400 1 2 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGGTG Intron 1 400 1 300 1 2 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTGGCAT Intron 1 400 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 <td></td> <td>SVi0-58B</td> <td></td> <td>Exon 9</td> <td></td> <td></td>		SVi0-58B		Exon 9		
C Primer Name Intron 1 RHD-SSP Gene location Primer conc. P RHD-11-1042F GGGTGACGAGTGAAAACTCTAGTCTCGAT Intron 1 400 RHD-11-1042F GGGTGACGAGTGAAAACTCTAGCTCGAT Intron 1 400 RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 1 nRHD-17-783R GACAAACTCGGGTTCAAATCAGGAGACC Intron 1 400 1 nRHD-17-783R GACAAACTTGGGTTCAAATCAGGAGACC Intron 1 400 2 RHD-17-783R GACAAACTGGGTTCAAATCCAGGAGTC Intron 1 400 2 RHD-14-783R GACAAACTGGGTTCAAAGTCGGCG Intron 1-exon 3 300 1 3 RHD-845F GAGAAAAGGATTCTTGTTGAGATAACTGTCG Intron 1-exon 10 300 1 3 DEX10-SP-1358-as CAGTGCTGCCGGAACATTG Intron 1 1 1 300 1 8 Primer Name Intron sequencing, sequencing primer Gene location 1 300 1 9 ThTor 173F CAGCACAGTTGAGAGGGTGCA Intron 1 1 1		SYi9-119F	TCCAAGATCTCTTCCAATTCAG	Exon 10		
C Primer Name Intron 1 RHD-SSP Gene location (nm0/L) leng RHD-11-1042F GGGTGACGAGTGAAACTCTATCTCGGAT Intron 1 400 400 Photential GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 400 D Primer Name Breakpoint long-range PCR primer Gene location (nm0/L) leng 1 nRHD-11-1405F CATTTCCCCTATTTAACAGACAAGAACAG Intron 1 400 400 2 RHD-11-1042F GGGTGACGAGTGAAACTCTGGCTCAATCAGGAGATC Intron 1 400 400 2 2 RHD-11-1042F GGGTGACGAGTGAAACTCTGTCGCAT Intron 1 400 400 1 1 RD-11-1042F GGGTGACGAGTGAAACTCTATCTCGCAT Intron 1 400 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1 300 1			100/04/10101100/04/10/04		Drimor conc	Droduct
RHD-i1-1042F GGGTGACGAGTGAAACTCTATCTCGAT Intron 1 400 RHD-i1-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 Primer Name Breakpoint long-range PCR primer Gene location Primer conc. P 1 nRHD_i1-1405F CATTTCCCCTATTTAACAGACAAGAACAAG Intron 1 400 2 RHD-i1-042F GGGTGACGAGTGAAACATCTATCTCGAT Intron 1 400 2 RHD-i1-042F GGGTGACGAGTGAAAACTCTATCTCGAT Intron 1 400 2 RHD-i1-042F GGGTGACGAGCATTCCTCAGGTTGCC Intron 1 400 3 RHD-i8-88F GAGAAAAGCATCTCTCAGGTTGCC Intron 1 400 3 DEX10-SP-1358-as CAGTGCCTGCGGGAACATTG Intron 1 400 3 DEX10-SP-1358-as CAGGCATGAGAACAAGAACAAGAACAAGAGG Intron 1 400 4 RD-i1-1398F AACAGACAAGAACAAGAACAAGAAGGG Intron 1 400 400 400 4 RD-i2+9458F AACGTGTATAGCTGTCCC Intron 1 400 400 400 400 4 RD-i2+945	С	Primer Name	Intron 1 RHD-SSP	Gene location	(nmol/L)	length (bp)
RHD-11-783R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 D Primer Name Breakpoint long-range PCR primer Gene location (nmol/L) len 1 nRHD_11-1405F CATTTCCCCTATTTAACAGACAAGAACAAG Intron 1 400 2 RHD-11-7838 GACAAACTTGGGTTCAAATCAGGACATC Intron 1 400 2 RHD-11-1042F GGGTGACGAGTGAAACTCTATCTCGCAT Intron 1 -exon 3 300 2 2 RS3-US-451-as ACTGATGACCATCCTCAGGTTGCC Intron 1 -exon 10 300 300 3 3 DEX10-SP-1358-as CAGTGCCTGGGCAGAACATTG Intron 1 -exon 10 300 300 3 E Primer Name Intron sequencing , sequencing primer Gene location 1 RD-11-788F CACACAGTTAGAGGAGTGCCA Intron 1 800 300 3 300 3 300 3 300 300 300 300 300 300 300 300 300 300 300 300 300 300 300 300 300 300 <	-	RHD-i1-1042F	GGGTGACGAGTGAAACTCTATCTCGAT	Intron 1	400	306
D Primer Name Breakpoint long-range PCR primer Gene location (mmol/L) len 1 nRHD_i1-1405F CATTTCCCCTATTTAACAGACAAGAACAAG Intron 1 400 2 RHD-i1-783R GACAAACTTGGGTTCAAATCAGGAGATC Intron 1 400 2 RHD-i1-1042F GGGTGACGAGTGAAACTCTATCTCGAT Intron 1		RHD-i1-783R	GACAAACTTGGGTTCAAATCAGGAGTC	Intron 1	400	
D Printer Name Breakpoint fong-targe PCA primer Gene location (mmor) 1 nRHD_i1-1405F CATTTCCCCTATTTAACAGACAAGAACAG Intron 1 400 2 RHD-i1-1042F GGGTGACGAGGACAACTTCAGCTCAGCAT Intron 1 400 2 EX3-US-451-as ACTGATGACCATCTCAGGTTCAAATCAGGAT Intron 1-exon 3 300 3 RHD-i8-88F GAGAAAAGGATTTCTGTTGAGATACTGTCG Intron 1-exon 10 300 3 DEX10-SP-1358-as CAGTGCCTGCGGGAACATTG Intron 1-exon 10 300 6 Primer Name Intron sequencing, sequencing primer Gene location 7 RD-i1-1398F AACAGACAAGAACAAGAAGAGG Intron 1 8 RD-i1-1398F CACACAGTTAGGGGGCCC Intron 1 8 RD-i1-1398F CACACAGTTAGAGGGGGG Intron 1 8 RD-i1-1398F CACACAGTTAGGGGAGGGGG Intron 1 8 RD-i2+486F GTTACCTGGGCAGAGGGGGGG Intron 2 8 RD-i2+945R AACGTGATATCCCC Intron 2 8 RD-i2+480F GCTGGGCACAGGGGGGGG Intron 2 8 RD-i2+480F GCTGGGCATGGTGGTTCA Intron 2 8 RD-i2+3083F AGTGCAGTGATCATTAGCT Intron 9 9 RDi9-4795F CCAGTCAGTGACATT			Dracks sist long some DOD primer	Conc location	Primer conc.	Product
1 nHHD_11-1405F CATTTCCCCTATTAACAGACAAGAG Intron 1 400 1 RHD-11-183R GACAAACTTGGGTTCAAATCAGGAGTC Intron 1 400 2 RHD-11-1042F GGGTGACGAGTGAAACTCTACAGGAGTC Intron 1 -exon 3 300 2 EX3-US-451-as ACTGATGACCATCCTCAGGTTGCC Intron 1 -exon 10 300 3 RHD-i8-88F GAGAAAAAGGATTTCTGTTGAGATACTGTC Intron 1 -exon 10 300 5 Primer Name Intron sequencing, sequencing primer Gene location 8 RD-i1-1398F AACAGACAAGAACAAGAACAAGAAGAG Intron 1 8 RD-i1-178F CAGCACAGTTAGAGGTGCCA Intron 1 8 RD-i1-788F CACACAGTTAGAGGTGCCA Intron 1 8 RD-i2+1480F GATCCTGGGCAGGTGGTCCA Intron 1 8 RD-i2+1480F GATTACTCGGGCAGGGTGGTCA Intron 2 8 RD-i2+1480F GAGTTAATTCCCACCGGGATCC Intron 2 8 RD-i2+1480F GAGTGACTGTGTCTCA Intron 2 8 RD-i2+1480F GAGTGACAGTGGTCCACATTGAC Intron 2 8 RD-i2+303F AGTGCAGTGGTACACATG	D	Primer Name	Breakpoint long-range PCR primer	Gene location	(nmoi/L)	length (bp)
1 HHD-11-/283H GACAAACT I GGG I I CAAAI CAGGAG I C Intron 1 400 2 RHD-11-/042F GGGTGACGAGTGACATTATCTCGAT Intron 1-exon 3 300 2 EX3-US-451-as ACTGATGACGAGTGGAAACTCTATCTCGGT Intron 1-exon 3 300 3 RHD-18-88F GAGAAAAAGGATTTCTGTTGAGATACTGTCG Intron 1-exon 10 300 3 DEX10-SP-1358-as CAGTGCCTGCGCGAACATTG Intron 1-exon 10 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-11-1398F AACAGACAAGAACAAGAACAAGGA Intron 1 RD-11-788F CACACAGTTAGAGGGTGCCA Intron 1 RD-11-788F CACACAGTTATGTTATCCCC Intron 1 RD-12+308F AACGTGATAAGCTTGATGTG Intron 2 RD-12+480F GCTGGGCATGGTGGTGCA Intron 2 RD-12+308JF GAGTGATGTCAGTGTGGC Intron 2 RD-12+308JF GAGTGAATCCCACGGGATGTG Intron 2 RD-12+308JF GAGTGCATGCTGCCAGGAGTGG Intron 2 RD-12+308JF GAGTGCATGCTGCCCC Intron 2 RD-12+308JF CACTCCTCCTCGTGCCCTTTG Intron 9 RD-12+308JF <td< td=""><td>1</td><td>nRHD_11-1405F</td><td>CALLICCCCTALLIAACAGACAAGAACAAG</td><td>Intron 1</td><td>400</td><td>670</td></td<>	1	nRHD_11-1405F	CALLICCCCTALLIAACAGACAAGAACAAG	Intron 1	400	670
2 HHD-11-1042F GGGTGACGAGTGCAACTCTACGGAT Intron 1-exon 3 300 2 EX3-US-451-as ACTGATGACCATCCTCAGGGTTGCC Intron 1-exon 10 300 3 DEX10-SP-1358-as CAGTGCCTGCGCGAACATTG Intron 1-exon 10 300 6 Primer Name Intron sequencing, sequencing primer Gene location 7 RD-i1-1398F AACAGACAAGAACAAGAAGAGG Intron 1 8 RD-i1-1398F AACAGACAAGAACAAGAAGAGG Intron 1 9 RD-i1-738F CACACAGTTAGGGGTGCCA Intron 1 9 RD-i2+945R AACGGCATGTTATGTTATCCCC Intron 1 9 RD-i2+945R AACGTGGATGGTGGTGGT Intron 2 9 RD-i2+866F GTTACCTGGGCATGGTGGTTCA Intron 2 9 RD-i2+2403F GAGTAATCCTCCTCGGTCACCTATGC Intron 2 9 RD-i2+3083F AGTGCAGTGATAATCCTCAGGGTTGC Intron 9 9 RDi9-4795F CCAGCTCAGTCACTTCAGGTGGTGCC Intron 9 9 RDi9-4226F CATATTGCAAGCTAAGGTGAGGCC Intron 9 9 RDi9-4226F CATATTGCAGTGAGCACGAGG Intron 9 9 RDi9-4226F <td>1</td> <td>RHD-11-783R</td> <td>GACAAACTIGGGTTCAAATCAGGAGTC</td> <td>Intron 1</td> <td>400</td> <td>7000</td>	1	RHD-11-783R	GACAAACTIGGGTTCAAATCAGGAGTC	Intron 1	400	7000
2 EX3-05-451-38 ACTGATGACCATCGTCAGGTTCAGATACTGTCG Intron 1-exon 10 300 3 DEX10-SP-1358-as CAGTGCCTGCGCGAACATTG Intron 1-exon 10 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-i1-1398F AACAGACAAGAACAAGAAGAGG Intron 1 RD-i1-788F CACACAGTTAGGTGTATGTTATCCCC Intron 1 RD-i2-945R AACGTGTATAGCTTGATGTG Intron 2 RD-i2-24945R GACGTGTATGGTGGG Intron 2 RD-i2-24945R GACGTGTATGGTGGG Intron 2 RD-i2-2403F GAGTGACAGGGGGGGGGG Intron 2 RD-i2+480F GCTGGGCAGGGGGACGATGCC Intron 2 RD-i2+2403F GAGTCAATCATAGC Intron 2 RD-i2+2403F GAGGCAGTGGTACAATCATAGC Intron 9 RD-i2+2403F CAGTCACTGCACTGGAGTTCC Intron 9 RD-i2+2403F CAGTCACTGCACTGCACTTGAGTTCC Intron 9 RDi9-5537F CCACTCCTCCTCTTG Intron 9 RDi9-4226F CATATTGCAAGTGAAGCCTACATGTAGT Intron 9 RDi9-42983R AATTAGCCGTGGTGCAGGG Intron 9 RDi9+2077R GCCAATGAAGAGATCTTCAGCAC Int	2	RHD-11-1042F	GGGTGACGAGTGAAACTCTATCTCGAT	Intron 1–exon 3	300	7382
3 DEX10-SP-1358-as CAGTGCCTGCGCGAACATTG Intron 10 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-11-1398F AACAGACAAGAACAAGAAGAGGG Intron 1 RD-11-788F CACACAGTTAGAGGGTGCCA Intron 1 RD-11-788F CACACAGTTAGAGGGTGCCA Intron 1 RD-12-945R AACCGTGTTATGTTATCCCC Intron 2 RD-12-945R AACCGGCAGGAGGTGG Intron 2 RD-12-945F GAGTTAATGCTTGATGTG Intron 2 RD-12-945F GAGTTAATTCCCACCGGGAGTGG Intron 2 RD-12-3083F GAGTTAATTCCCACCGGGATTC Intron 2 RD-12-3083F AGTGCAGTGGTACATCATAGCC Intron 9 RD19-4795F CCAGCTCAGTCACTTGAGTTGC Intron 9 RD19-4795F CCAGCTCAGTGACATCAAGGCT Intron 9 RD19-4226F CATTATGCCAGGTGGAGGG Intron 9 RD19-42283R AATTAGCCGTGGAGCAG Intron 9 RD19-2007R GCAATGGATGCATTGAGCCC Intron 9 RD19-210F GAAGTGCATGTCACTTGAGAGCC Intron 9 RD19-2216F AGAGTGCATGTCAGTGAGAGC Intron 9 RD19-2216F GAGGTG	2	EX3-US-451-as		Intron 1-exon 3	300	7075
S DEXT0-SP-13SP-2S CACIFICCUTICCUCCUCACATURG Inition 1-ext0110 300 E Primer Name Intron sequencing, sequencing primer Gene location RD-11-1398F AACAGACAAGAACAAGAAGAGG Intron 1 RD-11-788F CACACAGTTAGGGTGCCA Intron 1 RD-11-778F CGCATGTTATGTTATCCCC Intron 1 RD-12+945R AACGTGATATGCTTGATGTG Intron 2 RD-12+866F GTTACCTGGGCAGGAGGTGG Intron 2 RD-12+1480F GCATGTTAGGTGTCA Intron 2 RD-12+3083F AGTGCAGTGGTACAATCATAGC Intron 2 RD-12+3083F AGTGCATGGTGATCAATCATAGC Intron 2 RD-12+3083F AGTGCAGTGGTACAATCATAGC Intron 2 RD-12+3083F AGTGCAGTGGTGCCACTGGTGGTCC Intron 9 RD19-4295F CAAGTTCATCCTCCTGGTGCACGC Intron 9 RD19-4296F CAAGTGCATGTCAGTGAGCCC Intron 9 RD19+2206F CATATGCAGGAGAGGAGGCCC Intron 9 RD19+22007R GCCAATGGATGATCTCAGGCACC Intron 9 RD19-2216F AGGGGGGGGCATCTGCAAGG Intron 9 RD19-2216F AGGTGGGGGAGGCATCTGCAAGG Intron 9 RD19-	3		GAGAAAAAGGATTCTGTTGAGATACTGTCG	Intron 1-exon 10	300	1215
EPrimer NameIntron sequencing, sequencing primerGene locationRD-i1-1398FAACAGACAAGAACAAGAAGAGGIntron 1RD-i1-788FCACACAGTTAGAGGGTGCCAIntron 1RD-i1-778FCGGCATGTTATGTTATCCCCIntron 1RD-i2+945RAACGTGATAGCTTGATGTGIntron 2RD-i2+866FGTTACCTGGGCAGGAGGGTGGIntron 2RD-i2+1480FGCTGGGCATGGTGGTGCAIntron 2RD-i2+2403FGAGTTAATTCCCACCGGGATTCIntron 2RD-i2+3083FAGTGCAGTGGTACAATCATAGCIntron 9RDi9-5537FCACTTCCTCTGCTCCTTTGIntron 9RDi9-4296FCATATTGCAAGCTGAGTGGTGCAGGIntron 9RDi9-4296FCATATTGCAAGCCTACAGTTTCIntron 9RDi9-4226FCATATTGCAAGTCAGTGAGCCCIntron 9RDi9-2207RGCCAATGGAGGAGCACCIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTGCAGTGAGCCCAGGIntron 9RDi9-1495FGGAGTGGTGCAGTGAGCCGAGGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGGIntron 9RDi9-188PFGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGGIntron 9RDi9-162FGCATTTAAACAGGTTTGCCIntron 9RDi9-188PFGAAGATCATCCATGCCAGCGCGAGIntron 9RDi9-182FGAGGCAGAATTGCTTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTG	3	DEXT0-SP-1358-as	CAGIGCCIGCGCGAACAIIG		300	
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InternationCorrectionInternationRD-11-173FCGGCATGTTATGTCTCCCIntron 1RD-12+945RAACGTGTATAGCTTGATGTGIntron 2RD-12+945RGCTGGGCATGGTGGTTCAIntron 2RD-12+1480FGCTGGGCATGGTGGTTCAIntron 2RD-12+2403FGAGTTAATTCCCACCGGGATTCIntron 2RD-12+3083FAGTGCAGTGGTACAATCATAGCIntron 9RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACTGAGTTGCIntron 9RDi9+226FCATATTGCAAGCCTACAGTGTGCCIntron 9RDi9+2983RAATTAGCCGTGGTGGCACAGIntron 9RDi9+2907RGCAATGGAGGAGCATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGAGACATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGAAGATCATCCATTGGCTCTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-1162FGCATTTAAACAGGTTGCCCIntron 9RDi9-1162FGCATTTAAACAGGTTGCCCIntron 9RDi9-188RGCTGAGTTCCACCACTGCCAGGCCIntron 9RDi9-188FGCGAGTTCCACCACTGCCAGCTCIntron 9RDi9-281FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		BD-i1-788E		Intron 1		
RD-12+945RAACGTGTATAGCTTGATGTGInton 1RD-12+945RAACGTGTATAGCTTGATGTGGIntron 2RD-12+1480FGCTGGGCATGGTGGTTCAIntron 2RD-12+2403FGAGTTAATTCCCACCGGGATTCIntron 2RD-12+3083FAGTGCAGTGGTACAATCATAGCIntron 9RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACATCAGGTGCCIntron 9RDi9-4226FCATATTGCAAGCCTACAGTTCIntron 9RDi9+283RAATAGCCGTGGTGGCAGGIntron 9RDi9+2907RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGAAGATCATCCATTGGCAGGCGAGGIntron 9RDi9-1989FGAGGTGGTTGCAGGTGGCGAGGIntron 9RDi9-1981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-842FGAGGCAGATTGCAGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		BD-i1-173E	CGGCATGTTATGTTATCCCC	Intron 1		
RD-12-866FGTTACCTGGGCAGGAGGTGGInton 2RD-i2+1480FGCTGGGCATGGTGGTTCAIntron 2RD-i2+2403FGAGTTAATTCCCACCGGGATTCIntron 2RD-i2+3083FAGTGCAGTGGTACAATCATAGCIntron 9RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACTTGAGTTGCIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2907RGCAATGGATGATCTTCAGCACIntron 9RDi9+2007RGCCAATGGAGGCATCTGCAAGIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGCAATGGATGTGCAGGGCAGGIntron 9RDi9-1989FGCAATTGAAGCCGAGGIntron 9RDi9-1985FGGGTTGCAGTGCAGGTGCCIntron 9RDi9-188FGCAGTGGTTGCAGTGCAGGCCGIntron 9RDi9-188FGCAGTGGTTGCAGTGCAGGCCCIntron 9RDi9-188FGCAGTGGTTGCAGTGCCGIntron 9RDi9-184FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-285RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCGGIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCGIntron 9		BD-i2+945B		Intron 2		
RD-12+1480FGCTGGGCATGGTGGTTCAInton 2RD-12+1480FGAGTTAATTCCCACCGGGATTCIntron 2RD-12+3083FAGTGCAGTGGTACAATCATAGCIntron 2RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACTTGAGTTGCIntron 9RDi9+426FCATATTGCCAGTGGTGGCAGGIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2907RGCAATGGATGCAGTGAGCAGCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTGCAGGTTGCAGGCAGGIntron 9RDi9-1889FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1889FGCAGTGGTTGCAGGCAGGCCIntron 9RDi9-188FGGAGTGGTTGCAGTGCAGGTCCIntron 9RDi9-188FGCAGTGGTTGCAGGCAGGCCIntron 9RDi9-188FGAGGTGCTTCGAAGTCGCCGAGIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-785FGAGGCAGAATTGCTTGAAGTGGTGCCIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGGIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCGGIntron 9RDi9-7		BD-i2-866E	GTTACCTGGGCAGGAGGTGG	Intron 2		
RD-12+2403FGAGTTAATTCCCACCGGGATTCIntron 2RD-i2+3083FAGTGCAGTGGTACAATCATAGCIntron 2RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCCAGTCACTTGAGTTGCIntron 9RDi9+4226FCATATTGCAAGCCTACAGTGAGGIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2907RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGCAATGGATGATGTGCAGGCCGAGIntron 9RDi9-1495FGGATTGCAGTGAGCCGAGGIntron 9RDi9-188FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-188FGGAGTGGTTCCACCAGCGCCIntron 9RDi9-188FGAAGATCATCCATCGCAGGCCIntron 9RDi9-182FGAGGTTCCACCACTGCCAGCTCIntron 9RDi9-88FGAGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		BD-i2+1480F	GCTGGGCATGGTGGTTCA	Intron 2		
RD-12+3083FAGTGCAGTGGTACAATCATAGCIntron 2RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACTTGAGTTGCIntron 9RDi9-4226FCATATTGCAAGCCTACAGTTTCIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2983RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2907RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGGTAGCCGAGIntron 9RDi9-188FGAAGATCATCCACTGCCAGCTCIntron 9RDi9-188FGGAGTGGTTGCAGGCGAGCCCIntron 9RDi9-188FGCAGTGGTTGCAGCTGCCGAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-785FGAGGCAGAATTGCTTGAACGCGTGCCIntron 9RDi9-785FGAGGCAGAATTGCTTGAACCCGIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		BD-i2+2403F	GAGTTAATTCCCACCGGGATTC	Intron 2		
RDi9-5537FCACTTCCTCCTGCTCCTTTGIntron 9RDi9-4795FCCAGCTCAGTCACTTGAGTTGCIntron 9RDi9-4226FCATATTGCCAGCGCAGCAGTTTCIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2983RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2907RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2007RGCCAATGGAGGCACCIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGGAGTGGTGCAGGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RD-i2+3083F	AGTGCAGTGGTACAATCATAGC	Intron 2		
RDi9-4795FCCAGCTCAGTCACTTGAGTTGCIntron 9RDi9-4226FCATATTGCAAGCCTACAGTTTCIntron 9RDi9-2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2710RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1989FGAAGATCATCCATTGGCGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTGCCAGTGAGCCGAGIntron 9RDi9-1881FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-5537F	CACTTCCTCCTGCTCCTTTG	Intron 9		
RDi9-4226FCATATTGCAAGCCTACAGTTTCIntron 9RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2710RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCCAGGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCCCIntron 9RDi9-162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-4795F	CCAGCTCAGTCACTTGAGTTGC	Intron 9		
RDi9+2983RAATTAGCCGTGGTGGCAGGIntron 9RDi9+2710RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCAGGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCCGAGIntron 9RDi9-162FGCATTTAAACAGGTTTGCCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-4226F	CATATTGCAAGCCTACAGTTTC	Intron 9		
RDi9+2710RGAAGTGCATGTCAGTGAGCCCIntron 9RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCAGCIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9+2983R	AATTAGCCGTGGTGGCAGG	Intron 9		
RDi9+2007RGCCAATGGATGATCTTCAGCACIntron 9RDi9-2216FAGGTGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-785RGGGTTCCACCAGCTGCAGCTCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCGGIntron 9		RDi9+2710R	GAAGTGCATGTCAGTGAGCCC	Intron 9		
RDi9-2216FAGGTGGGGAGCATCTGCAAGIntron 9RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9+2007R	GCCAATGGATGATCTTCAGCAC	Intron 9		
RDi9-1989FGAAGATCATCCATTGGCTTCTGIntron 9RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-2216F	AGGTGGGGAGCATCTGCAAG	Intron 9		
RDi9-1495FGGAGTGGTTGCAGTGAGCCGAGIntron 9RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-1989F	GAAGATCATCCATTGGCTTCTG	Intron 9		
RDi9-1162FGCATTTAAACAGGTTTGCTCCIntron 9RDi9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-1495F	GGAGTGGTTGCAGTGAGCCGAG	Intron 9		
HDI9-981FGGGTTCCACCACTGCCAGCTCIntron 9RDi9-785RGCTGAGTTCTGAAGTGGTGCCIntron 9RDi9-482FGAGGCAGAATTGCTTGAACCCGIntron 9		RDi9-1162F	GCATTTAAACAGGTTTGCTCC	Intron 9		
RDI9-785R GCTGAGTTCTGAAGTGGTGCC Intron 9 RDI9-482F GAGGCAGAATTGCTTGAACCCG Intron 9		RDi9-981F	GGGTTCCACCACTGCCAGCTC	Intron 9		
HD19-402F GAGGGAGAATIGGTIGAAGGGG INTON 9		HD19-785H	GUIGAGITUTGAAGIGGIGUU	Intron 9		
		KU19-482F	GAGGUAGAATIGUTIGAAUUUG	INTON A		

The presence for anti-LW^a in the serum sample of the weak D type 26–immunized mother was tested by comparing adsorption of the antibody in question to three samples each of D+ and D– cord and adult blood cells.²⁷

Population genetics and statistical analysis

Haplotype frequencies of cde, Cde, and cdE were calculated from an appropriate number of donors from the respective transfusion centers in Innsbruck, Oldenburg, and Ljubljana. The haplotype associations of the respective *RHD* genes with either the haplotype Cde or the haplotype cdE were apparent. *RHD* allele frequencies were calculated from their frequencies in the respective haplotype and the calculated frequency of these haplotypes in the local population. Confidence intervals (CIs) were calculated according to the Poisson distribution.²⁸ Donors were not checked for kinship.

Contributions

Sample collection, serologic typing, and DNA preparation was performed in each participating laboratory. Serologic retesting of all Kirov samples was performed in Göttingen, Germany. PCR screening with three PCR-SSP reactions and RHD exon scanning was performed in Innsbruck for samples from Innsbruck, Bern, Kirov, and Braunschweig and in Oldenburg (with capillary electrophoresis for exon scanning) and Ljubljana. DNA sequencing for all but the Oldenburg samples was performed in Innsbruck, including two samples from Oldenburg, which were sequenced in Oldenburg and Innsbruck, but did not show any mutation(s). Flow cytometry analysis of weak D type 26 was performed in Vienna.

RESULTS

Blood transfusion centers from Innsbruck, Oldenburg, Ljubljana, Bern, Kirov, and Braunschweig contributed 738, 400, 333, 104, 71, and 54 samples, respectively. All 1700 samples were from blood donors typed D– by serology but C+ and/or E+ (Table 2A). They were collected for laboratory-dependent time frames in 2002 and 2003 by chance, with an exception from Bern, which overweighted its collection of samples with E.

DNA samples were screened individually by three PCR-SSP procedures specific for the *RHD* 5'-untranslated region and exons 3 and 10. All 89 partially and complete *RHD+* samples among D– individuals were then analyzed further with an *RHD* exon scanning PCR-SSP, specific for *RHD* exons 3, 4, 5, 6, 7, 9, and 10 and an additional PCR-SSP system with additional specificities for the *RHD* 5'-untranslated region and exons 2 and 8. The absence of *RHD*-specific amplification products led to the recognition of 1 *RHD* category VI type 1 sample and 59 *RHD-CE*-

D hybrid alleles. With respect to these hybrid alleles, partial deletions of the RHD gene, or heterozygous combinations of different RHD-CE-D hybrid alleles, could not be excluded formally at this stage of investigation. The remaining 30 samples with the presence of all RHDspecific amplification products were further analyzed with five PCR-SSP procedures, capable of properly identifying weak D types 1 to 5. This approach yielded 1 weak D type 1 and 2 weak D type 5. The remaining 25 samples with the RHD signal were DNA sequenced for all 10 RHD exons including parts of the adjacent introns: among these, 1 novel weak D type 26 allele, 1 novel DEL allele RHD(X418L), 8 DEL RHD(M295I), 6 DEL RHD(IVS3+1G>A), and 1 novel unexpressed RHD(Y401X) allele, together with 9 unexpressed RHD alleles, of which no molecular cause could be recognized. Summarized results are shown in Table 2C.

RHD-CE-(D) hybrid alleles

A total of 59 *RHD-CE-(D)* hybrid alleles were identified. The majority of samples with any positive *RHD*-specific PCR-SSP procedures were *RHD-CE-D* hybrid alleles with large parts of the *RHCE* gene, for example, 39 *RHD-CE(2-9)-D*, 13 *RHD-CE(2-10)*, 4 *RHD-CE(2-8)-D*, 2 *RHD-CE(4-8)-D*, and 1 *RHD-CE(4-7)-D*. The alleles *RHD-CE(2-10)*, *RHD-CE(2-8)-D*, and *RHD-CE(4-8)-D* were new alleles and had not been reported previously.

With respect to *RHD-CE(2-10)*, the claimed specificity for the *RHD* 5'-untranslated region—defined only by one PCR-SSP procedure specific for adenine, positioned 132 nucleotides in front of the start codon ATG (please refer to reaction 1 of Table 1A)—was further investigated by the analysis of *RHD* zygosity, for example, testing the presence of an *RHD* hybrid box in these cases. All above-mentioned samples showed *RHD* hybrid box homozygosity, indicating that there was no *RHD* gene at all, but rather an *RHCE* variant allele with A-132 in its genomic DNA sequence.³

For all these alleles, PCR-SSP characterization was definitive, beside the most common, which were *RHD*-*CE*(2-9)-*D* and *RHD*-*CE*(2-8)-*D*. Because of their DNA sequence similarity, unambiguous PCR-SSP typing for *RHD* exon 2 in the presence of *RHC* is blocked. Analysis of 34 of all 39 *RHD*-*CE*(2-9)-*D* hybrid alleles identified in this study (only lacking 3 samples from Oldenburg and 2 samples from Ljubljana) with a PCR-SSP detecting *RHD*-specific adenine, however, 1038 bp 5' of the intron 1–exon 2 boundary, showed positivity in all cases (Fig. 2). No evidence for allelic subgroups, for example, "D1 and D2"—as described earlier—could be observed.¹⁹

DNA sequencing of the 3' part of intron 1 and exon 2, the 5' part of intron 2, and the 3' part of intron 9 was carried out of a *RHD-CE(2-9)-D* sample to define possible breakpoints. Sequences were deposited under AJ633649 for intron 1 and exon 2, AJ633650 for intron 2, and

	IBK	OLD	LJU	BER	KIR	BRA	SUM
A. Number of phenotypes investigated							
C – dd E –	0	0	0	1	0	0	1
C c dd E –	0	0	0	0	0	0	0
– c dd E –	3	3	0	0	0	0	6
C – dd E e	0	0	0	1	0	0	1
C c dd E e	7	0	1	3	0	0	11
- c dd E e	189	125	36	50	5	17	422
C - dd - e	9	5	12	3	2	1	32
C c dd – e	530	267	284	46	64	36	1227
Total	738	400	333	104	71	54	1700
B. Rh haplotypes included							
C - d - e	555	277	309	56	68	38	1303
– c d E -	202	131	37	53	5	17	445
C – d E – (assumption: no CdE in D– C+ c– E+ e+ sample)	0	0	0	3	0	0	3
cd-e	719	392	320	96	69	53	1649
Total	1476	800	666	208	142	108	3400
C. All samples with RHD gene							
C c dd – e. <i>RHD-CE(2-10)-D</i>	0	0	13	0	0	0	13
C c dd – e, <i>RHD-CE(2-9)-D</i>	29	3	4	1	0	2	39
C c dd – e, new allele RHD-CE(2-8)-D	0	4	0	0	0	0	4
C c dd – e, new allele RHD-CE(4-8)-D	0	1	1	0	0	0	2
– c dd E e, <i>RHD-CE(4-7)-D</i>	1	0	0	0	0	0	1
C c dd – e, DEL <i>RHD</i> (M295I)	5	1	1	0	0	1	8
C c dd – e, DEL <i>RHD</i> (IVS3+1G > A)	1	0	3	2	0	0	6
C c dd – e, new allele DEL 1252T(Tins)1253 A(X418L)†	1	0	0	0	0	0	1
– c dd E e, new allele D– T1203A(Y400X)‡	0	0	0	0	1	0	1
 – c dd E e, RHD category VI type 1 	1	0	0	0	0	0	1
C c dd – e, weak D type 1	0	0	1	0	0	0	1
– c dd E e, weak D type 5	2	0	0	0	0	0	2
C – dd – e, new allele weak D type 26 T26A (V9D)§	1	0	0	0	0	0	1
C c dd – e, regular RHD ?	0	8	1	0	0	0	9
Total	41	17	24	3	1	3	89

* A shows the number of different Rhesus phenotypes investigated from each participating transfusion center: Innsbruck, Austria (IBK); Oldenburg, Germany (OLD); Ljubljana, Slovenia (LJU); Bern, Switzerland (BER); Kirov, Russia (KIR); and Braunschweig, Germany (BRA). B gives the numbers of respective Rh haplotypes investigated. For all D- C+ c+ E+ e+ samples, heterozygosity of Rh haplotypes Cde/cdE was assumed. C shows all single observations of *RHD*+ alleles identified in the various participating transfusion centers. For all panels, on the left-hand side, Rh phenotypes (haplotypes) are indicated, and the column on the far right gives the total sum for each observation of all participating transfusion centers. Total sums for each transfusion center are given at the bottom of each panel.

† Insertion of T at coding nucleotide 1253 (exon 10).

‡ Substitution of T1203A (Y401X) premature STOP (exon 9).

§ Substitution of T26A (V9D) (exon 1).

AJ633651 for intron 9. RHD and RHCE genomic sequences were compared to respective DNA sequences from genomic contigs AL928711 and BX640519. The 5' breakpoint area showed an RHD/RHC-specific sequence until an RHC-specific 109-bp insertion in intron 2 of RHD-CE(2-9)-D. Interestingly, RHD-CE(2-9)-D shows one specific nucleotide exchange at coding nucleotide 203, which is changed from G in RHD/RHC to A, giving rise to a predicted amino acid exchange from serine to threonine at amino acid position 68. The deduction of the 3' breakpoint in RHD-CE(2-9)-D was ambiguous, because nucleotide position -4359 suggests the most 5'-located RHD-specific nucleotide in intron 9 of RHD-CE(2-9)-D, whereas the further 3'-located nucleotide position -4159 shows RHCE specificity again, before exon 10 cDNA nucleotide position 1359 (and following nucleotides) finally exhibited RHD

specificity. Nucleotide positions –4757 and –1862 were specific for *RHD-CE(2-9)-D* (Fig. 3).

Weak D type 26

During the fourth pregnancy of a D– C+ c– E– e+ woman (with documented anti-D– throughout her previous three pregnancies), anti-D alloantibody formation was observed after having received 3 units of RBCs of D– C+ c– E– e+ phenotype. The presence of anti-LW^a was excluded by demonstrating complete adsorption of the antibody in question to D+ but not to D– cord blood cells. The husband of the mother typed D– C– c+ E– e+ in serology and DNA; all children were of D– C+ c+ E– e+ phenotype as shown by serologic methods (including IAT). Further investigation of the donations revealed two *RHD* DNA–



Fig. 2. PCR-SSP analysis of *RHD-CE*(2-9)-*D* hybrid alleles PCR-SSP analysis of 34 of 39 *RHD-CE*(2-9)-*D* hybrid alleles is shown. The presence of *RHD*-specific adenine located 1038 bp 5' of the intron 1–exon 2 boundary gives rise to a 306-bp *RHD*-specific amplification product, which can be identified in all *RHD*-*CE*(2-9)-*D* samples investigated. RH DNA types of the following samples served as controls: 2 D– C– c+ E– e+ (A), 2 D+ C+ c+ E– e+ (B), 2 D+ C– c+ E– e+ (C), and 2 D– C+ c+ E– e+ (D). In each reaction, a 434-bp control band was coamplified as a positive amplification control.

positive RBC units among the three given. One of them-IBK376-had already been included in the presented study (historical record). Both RHD+ donor samples represented siblings; their weak D were originally overseen by routine IAT performed in tubes, but could clearly be shown to be positive when retested with a gel matrix IAT system. DNA sequencing of the entire RHD gene of both samples revealed a changed nucleotide position 26 from T in RHD to A, leading to an amino acid exchange at an amino acid position 9 from valine to aspartate in the observed allele. The amino acid exchange was predicted to be located intracellular on the D peptide and is therefore unlikely to behave as a partial RHD. Consequently the new allele was named weak D type 26; its exon 1 DNA sequence was deposited under EMBL Accession Number AJ534720.

D serology from both the related donors showed the presence of a weak D, as shown by weak positive reactions in IAT. D antigen density was determined and differed in the female and the male (IBK376) sample, which showed 29 and 70, compared to 10.215 and 19.770, D antigens per cell in D+ C+ c+ E– e+ and D+ C+ c– E– e+ RBCs, respectively. The obvious difference in D antigen numbers between the two weak D type 26 individuals was also evident when using anti-D typing reagents in IAT in gel matrix. Here, one weak D type 26 sample was reactive with all anti-D reagents, whereas the other sample displayed consistently weaker or even negative reactions. These circumstances corroborated the flow cytometric results. Testing for *RHD* zygosity demonstrated the presence of

hybrid and upstream Rhesus box in both siblings and consequently excluded homozygosity for weak D type 26 in both samples. Thus, the more than twofold increase of D antigens per cell in the male sample (IBK376) could not be explained by an existing *RHD* gene dosage effect. The influence of *RHAG* could also be excluded, because both samples demonstrated regular RhAG expression.

DELs

With respect to the 15 DEL alleles observed in total, 6 were *RHD*(IVS3+1G>A), 8 were *RHD*(M295I), and 1 was a new DEL allele: *RHD*(X418L). The respective sample—IBK475—showed a negative IAT and positivity in the adsorption-elution test. The allele itself is caused by an insertion of a single T in between coding nucleotide 1252 and 1253 of exon 10 of the *RHD* gene ("1252T(Tins)1253A"), hereby causing a frameshift, which changed the previous *RHD* translation stop codon TAA(X) to TTA(L) leading to a predicted extension of the RhD protein from 417 to 488 amino acid. The DNA sequence of all 10 *RHD*(X418L) exons was deposited under EMBL Accession Numbers AJ630375 to AJ630384.

One sample with an unexpressed *RHD* gene with an identified mutation

Kirov sample KIR39—presenting an apparently regular RHD gene, but not showing any antigen D expression at all (negative IAT, negative adsorption-elution)—showed a transversion T1203A. This T1203 mutation changed coding sequence of codon 401 from TAT to TAA, and the respective amino acid sequence of the D peptide from a tyrosine to a stop codon, leading to a predicted premature stop of translation (Y401X). The DNA sequence of all 10 *RHD*(Y401X) exons was deposited under EMBL Accession Numbers AJ630385 to AJ630394.

Nine samples with an unexpressed *RHD* gene, but without identifiable mutation(s)

An additional nine samples could be identified without D expression, but an apparently intact *RHD* gene. DNA sequencing of all coding exons and adjacent intron sequences did not reveal any specific point mutation(s). A vast majority of these alleles—eight of nine—were observed in Oldenburg; only 1 of them was observed in Ljubljana. No additional DNA sequencing of intronic sequences was performed in these cases. The molecular reason for the lack of expression in these alleles could not be identified.

Transfusion reactions

Analysis of transfusion reactions to transfused RBC units was controlled for the 5 DEL *RHD*(M295I) individuals of

A: intron 1 exon 2	-1060	-1041	-1039	-1024	-994	-936	-804	-783	-778	-763	-658	-610	-482	-416	-335	-322	-201	-17	150	178	201	203	307
RHc	AA	Α	Α	A	Α	т	С	С	т	А	Α	т	Α	Т	А	Α	С	А	С	С	A	А	С
RHC	AA	А	А	А	А	т	т	G	С	G	G	С	G	С	G	G	т	G	т	А	G	G	т
RHD	-	G	т	-	G	С	т	G	с	G	G	с	G	с	G	G	т	G	т	А	G	G	т
RHD-CE(2-9)-D	-	G	т	-	G	С	т	G	С	G	G	С	G	С	G	G	т	G	т	А	G	С	т

B: intron 2	3021	3026	3087	3091	3205	3262	3265	3283	3296
RHc	A	π	С		С	т	G	G	G
RHC	т		т	109 bp	С	С	G	G	G
RHD	т		т		G	С	А	т	А
RHD-CE(2-9)-D	т		т	109 bp	С	С	G	G	G

C: intron 9 exon 10	-5443	-5369	-5083	-4757	-4359	-4159	-1862	1359-
RHCE	G	А	С	G	С	т	G	TTATGT
RHD	А	G	т	G	т	С	G	AATGTT
RHD-CE(2-9)-D	G	А	С	С	т	т	А	AATGTT

Fig. 3. Breakpoints in between genomic DNA sequences for RHD and RHCE for RHD-CE(2-9)-D. Intronic nucleotide positions are given relative to the respective exon-intron boundaries; exonic nucleotide positions are given according to RHD cDNA sequence. RHD-specific sequences are underlayed in gray, RHD-CE(2-9)-D-specific sequences are underlayed in black with white letters. (A) Informative polymorphisms for the RHD-CE(2-9)-D 5' breakpoint. It compares RHc, RHC, RHD, and RHD-CE(2-9)-D beginning 1060 bp 5' of intron 1-exon 2 boundary, ending with exon 2. RHD-CE(2-9)-D shows one specific nucleotide exchange at coding nucleotide 203, which is changed from G in RHD and RHC to A, giving rise to a predicted amino acid exchange from serine to threonine at amino acid position 68. (B) Informative polymorphisms for the RHD-CE(2–9)-D 5' breakpoint. It compares RHc, RHC, RHD and RHD-CE(2-9)-D beginning 3021 bp 3' of exon 2-intron 2 boundary, ending with nucleotide 3296. An RHC-specific 109-bp insertion is present in RHD-CE(2-9)-D at nucleotide position 3091. (C) Informative polymorphisms for the RHD-CE(2-9)-D 3' breakpoint. It compares RHCE, RHD, and RHD-CE(2-9)-D beginning 5443 bp 5' of intron 9-exon 10 boundary and ends with exon 10 cDNA position 1359. Deduction of the RHCE-RHD breakpoint is ambiguous, because nucleotide position -4359 suggests the most 5'-located RHD-specific nucleotide in intron 9 of RHD-CE(2-9)-D, whereas the further 3'-located nucleotide position -4159 shows RHCE specificity again, before exon 10 cDNA nucleotide position 1359 (and following nucleotides) finally exhibit RHD specificity. Nucleotide positions -4757 and -1862 are specific for RHD-CE(2-9)-D.

the sample group of 738 from Innsbruck (Austria). A total of 7 units of RBCs of the 5 donors were transfused to D-patients without previous knowledge, and their antibody status was checked by chance between 17 and 415 days after transfusion (mean, 141 days). No anti-D could be detected in these recipients by IAT. The other D+ RBC units from Innsbruck of individuals with *RHD* category VI type 1 and *RHD*(IVS3+1G>A) were not; weak D type 5 and *RHD*(X418L) was only transfused once to D– patients. Again, no D-specific antibodies could be detected in these recipients.

Population genetics of Innsbruck, Oldenburg, and Ljubljana

The Rhesus haplotype frequencies of cde, Cde, and cdE were calculated for Innsbruck, Oldenburg, and Ljubljana, which together contributed more than 86 percent (Table 2B). The frequency of Rhesus haplotype cde was almost identical in all three centers investigated (0.41999, 0.42228, and 0.41691), but differed considerably for Cde (0.01496, 0.00959, and 0.02022) and cdE (0.00686, 0.00382, and 0.00250), respectively.

Additional differences among the three transfusion centers were evident with respect to the regional-specific alleles of multiple occurrence: Regional exclusivity but no respective significance (p < 0.05) was given for 13 RHD-CE(2-10) in Ljubljana and 4 *RHD-CE*(2-8)-*D* in Oldenburg. The RHD(IVS3+1G>A) was only identified in the southern parts of the investigated area, for example, in the centers of Innsbruck and Ljubljana (also in Bern), but was completely lacking in the northern center of Oldenburg (also in Braunschweig and Kirov), again with no significance (p < 0.05). With respect to RHD-CE(2-9)-D, its frequency differed significantly in between Innsbruck and Oldenburg (p < 0.01), but did not for RHD(M295I). Estimated frequencies and respective 95 percent CIs for some alleles encountered in this study are given in Table 3.

DISCUSSION

This study was predominantly carried out to quantify the proportion of *RHD* positivity among serologically D– individuals, positive for antigens C and/or

E. The study should also help to assess the risk of alloimmunization against D after transfusing weakly expressed D+ RBC units to D- recipients. These weakly expressed D+ donations are usually missed by routine serologic methods and most commonly found among donors of the above-mentioned phenotype. Six central European blood transfusion centers participated in the collection and analysis of a total of 1700 samples, also providing important population genetics data.

New *RHD* alleles could be discovered in the course of this study, DEL *RHD*(X418L) with a predicted polypeptide

TABLE 3. Estimated population frequencies and 95 percent CIs of
selected RHD alleles found in Innsbruck (IBK), Oldenburg (OLD), and
Ljublana (LJU)*

			95% CI				
Alleles observed	Number	Estimate	Lower	Upper			
IBK							
RHD-CE(2-9)-D	29	1:640	1:453	1:974			
RHD-CE(2-8)	0	NO	1:5646	Infinite			
DEL RHD(M295I)	5	1:3710	1:1660	1:9415			
DEL RHD(IVS3+1G>A)	1	1:18550	1:3485	1:363667			
DEL RHD(K409K)	0	NO	1:5646	Infinite			
Weak D type 26	1	1:18550	1:3485	1:363667			
RHD unexpressed	0	NO	1:5646	Infinite			
OLD							
RHD-CE(2-9)-D	3	1:4817	1:1784	1:17663			
RHD-CE(2-8)	4	1:3613	1:1506	1:10577			
DEL RHD(M295I)	1	1:14449	1:2715	1:283297			
DEL RHD(IVS3+1G>A)	0	NO	1:4399	Infinite			
DEL RHD(K409K)	0	NO	1:4399	Infinite			
Weak D type 26	0	NO	1:4399	Infinite			
RHD unexpressed	8	1:1807	1:969	1:4399			
LJU							
RHD-CE(2-9)-D	4	1:1911	1:796	1:5594			
RHD-CE(2-8)	0	NO	1:2326	Infinite			
DEL RHD(M295I)	1	1:7641	1:1436	1:149823			
DEL RHD(IVS3+1G>A)	3	1:2547	1:943	1:9341			
DEL RHD(K409K)	0	NO	1:2326	Infinite			
Weak D type 26	0	NO	1:2326	Infinite			
RHD unexpressed	1	1:7641	1:1436	1:149823			
* Estimated population frequ	encies and lowe	er and upper limi	ts of the 95 pe	rcent CIs of			

selected *RHD* alleles of three of the participating transfusion centers are given. NO = not observed.

length of 488 amino acid and unexpressed NEX *RHD*(Y401X) with a predicted length of 400 amino acids being two of them.

For NEX *RHD*(Y401X), its complete lack of expression could be explained by the lack of D404 in the truncated protein. D404 of D corresponds to D399 of the AG protein as demonstrated by DNA and protein alignment analysis (data not shown). Lack of D399 in AG abolishes the association of AG to the D2 domain of ankyrin R, which further causes the lack of the Rh complex in the RBC membrane.²⁹ Therefore, the absence of a NEX *RHD*(Y401X) protein on the erythrocytes of the investigated individual could be explained by a comparable mechanism.

With respect to *RHD-CE(2-9)-D*, PCR-SSP analysis for *RHD* in intron 1 and genomic DNA sequencing of longrange PCR fragments did not show any evidence for two suballelic versions (D1 and D2), as proposed earlier.¹⁹ Interestingly, the sequenced example (and one other, of totally two sequenced in exon 2, data not shown) showed a nucleotide substitution at coding nucleotide 203, leading to a predicted S68T amino acid exchange. This location of the substitution would be predicted to be in the second intracellular domain of the respective protein and, therefore, would probably not exhibit any immunologic features. Owing to the conformational nature of the Rh epitopes, however, amino acid substitutions in a membrane-spanning domain may well contribute to phenotypes as exemplified by the VS antigen and its associated abnormal e expression.

The rationale of the intense study of the RHD-CE(2-9)-D samples was to improve *RHD* zygosity testing, because *RHD/RHD-CE(2-9)-D* heterozygous individuals would be wrongly interpreted as RHD/RHD homozygotes by currently used methods.^{3,31} In fact, RHD-CE(2-9)-D hybrid alleles do show an upstream box indicative for RHD (data not shown), but are actually D-. This becomes even more important when considering the relatively high frequency of RHD-CE(2-9)-D in the Caucasian population. The presented data can now be used to devise a RHD-CE(2-9)-D specific PCR-SSP to circumvent these problems.

Recently, a new *RHD-CE-D* hybrid allele—*RHD*(1)-*CE*(2-10)—has been described.³² But whereas "our" Caucasian *RHD*(1)-*CE*(2-10) is upstream box negative, the Shenzen *RHD*(1)-*CE*(2-10) shows this additional *RHD* specificity, clearly indicating a different molecular background for these two alleles.

Another novel RHD allele was weak D type 26, which proved to be clinically important. Respective RBC units had been transfused to a 24-years-old D- C+ c- Ee+ mother who had received two respective RBC units and subsequently developed a positive direct antiglobulin test, and alloanti-D. The two RHD+ donor samples represented siblings. In the course of this analysis, antigen density was determined to be in between 29 and 70 D antigens per RBC. Usually, transfusion of a relatively large amount of highly immunogenic D+ RBCs (200 mL or more) causes alloanti-D induction in more than 80 percent of the recipients within 2 to 5 months.³³ More recent data hint toward an inverse correlation between the number of transfused units and the probability of antibody formation, moving transfusion of minor amounts of D or weak D into the focus of interest.³⁴ The described finding of weak D type 26 is of particular interest, because the least amount of D antigens required for anti-D immunization is not known. Proven immunizations with low amounts of D antigens are those reported for weak D type 2 (antigen density of 450), for a D+ chimera (revealing 94% D- RBCs and an admixture of only 6 percent D+ RBCs) and examples of deliberate immunization of, for example, 5 mL of D+ RBCs.^{19,35,36} In the late 1940s and early 1950s, it was reported that blood of weak D phenotypes could cause anti-D alloimmunization in D– individuals, but the first detailed description with the defined causing molecular type involved was only published in 2000.^{27,35} The weak D type 26 described here, with its extremely low antigen density, is the weakest D known to cause anti-D immunization so far.

Our study uncovered a total of 15 DEL carriers, 1 of them showing a new DEL allele, RHD(X418L). RHD(M295I) was encountered eight times and RHD(IVS3+1G>A) six times. With these identifications of DEL carriers, we focused on the effect of DEL RBCs when transfused to D– individuals. All transfusions of DEL RHD(M295I) RBC units in the Innsbruck center were traced and analyzed. No anti-D immunization could be observed in the seven transfusion events investigated in total. This is of interest, because information about the general potential of DEL RBCs leading to anti-D immunization is scarce. Considering these data, we assumed, that anti-D immunization against DEL RBC is rare, if at all—at least for DEL RHD(M295I).

This study also includes facts about the economic aspect of the proposed diagnostic screening procedure. Because DNA preparation contributes significantly to the total costs, the preparation strategy has a big influence on final total costs. Methodically, blood samples of interest can be prepared individually or be pooled following various strategies, but with the disadvantageous delay of individual results. Taking 100 blood samples as the calculative number, however, approximately 50 DNA preparations (considering the "worst-case scenario" of a high frequency of RHD positives among D- and employing specific pooling regimens), screening of each individual, further PCR-SSP analysis of about 6, and finally RHD DNA sequencing of about 1 sample seem to be necessary and bring the total costs for 1 sample to approximately \$25 USD. With respect to the 738 samples investigated in the Innsbruck center, 1 anti-D immunization could have been avoided definitely by a timely screening procedure. An additional 3 samples—1 RHD category VI type 1 and 2 weak D type 5—were identified, which are thought to cause anti-D when transfused to D- recipients, because of their known D antigen density of 1050 and 296, respectively.²⁴ The category VI protein, however, lacks most of the D epitopes, which could severely reduce its immungenicity.³⁰ In any case, the respective samples should have been identified earlier by routine serologic methods. Therefore, to decide on the establishment of the proposed screening procedure for routine purposes, one influential variable is given by the quality of routine serologic methods, especially to identify weakly expressed D. Other variables can be national health policies and the level of priority in avoiding anti-D immunization. Nevertheless, the authors-at least-are strongly recommending the individual molecular diagnostic analysis of transfusion events, involving C or E homozygous D- donors.

Numerous studies have already been addressing RHD positivity among D- individuals. It is intriguing that regional differences in the relative composition of these groups in even nearby regions is generally encountered. This poses the important question as to which extent locally confined findings can be extrapolated to a complete ethnic group; for example, it seems precarious to claim Caucasian ("European") significance for a regional area. Comparing our findings to a previous study, significant differences in allele frequencies and expected "phenotype" frequencies could be observed, as exemplified by the D-alleles RHD-CE(2-9)-D, RHD-CE(2-8)-D, and a DEL RHD(K409K). Especially, RHD(K409K) was estimated to be encountered at a frequency of about once among 100 Cde haplotypes earlier, but could not be observed once among all 1303 Cde haplotypes investigated in this study.¹⁹ Further support for the need of a careful interpretation of population specific data comes from earlier reports on pronounced regional differences found among three Central European populations when investigating weak D.²³ Reliable and detailed population genetic data will have to be taken into consideration and used as a basis to devise generally acceptable molecular typing strategies for RHD in the near future.

ACKNOWLEDGMENTS

The help of EE Wagner, O. Herrmann, and U. Bauerfeind U (DRK Institute Springe, Germany) in generating data for the calculation of RH haplotype frequencies of the transfusion center of Oldenburg is acknowledged. The expert technical assistance of H.P. Spoetl and D. Koell of the Innsbruck transfusion center is appreciated. The support of H. Ulmer (Innsbruck Institute for Biostatistics and Documentation) for the help in the statistical analysis of data is acknowledged.

REFERENCES

- Colin Y, Cherif-Zahar B, Le Van Kim C, et al. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. Blood 1991;78:2747-52.
- Le van Kim C, Mouro I, Cherif-Zahar B, et al. Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci U S A 1992;89:10925-9.
- 3. Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. Blood 2000;95:3662-8.
- Wagner FF, Gassner C, Muller TH, et al. Molecular basis of weak D phenotypes. Blood 1999;93:385-93.
- 5. Hyland CA, Wolter LC, Saul A. Three unrelated Rh D gene polymorphisms identified among blood donors with Rhesus CCee (r'r') phenotypes. Blood 1994;84:321-4.
- 6. Carritt B, Steers FJ, Avent ND. Prenatal determination of fetal RhD type. Lancet 1994;344:205-6.

- Blunt T, Daniels G, Carritt B. Serotype switching in a partially deleted RHD gene. Vox Sang 1994;67:397-401.
- 8. Chang JG, Wang JC, Yang TY, et al. Human RhDel is caused by a deletion of 1,013 bp between introns 8 and 9 including exon 9 of RHD gene. Blood 1998;92:2602-4.
- Sun CF, Chou CS, Lai NC, Wang WT. RHD gene polymorphisms among RhD-negative Chinese in Taiwan. Vox Sang 1998;75:52-7.
- 10. Shao CP, Maas JH, Su YQ, Kohler M, Legler TJ. Molecular background of Rh D-positive, D-negative, D (el) and weak D phenotypes in Chinese. Vox Sang 2002;83:156-61.
- Huang CH. Alteration of RH gene structure and expression in human dCCee and DCW-red blood cells: phenotypic homozygosity versus genotypic heterozygosity. Blood 1996;88:2326-33.
- 12. Faas BH, Beckers EA, Simsik S, et al. Involvement of Ser103 of the Rh polypeptides in G epitope formation. Transfusion 1996;36:506-11.
- Avent ND, Martin PG, Armstrong-Fisher SS, et al. Evidence of genetic diversity underlying Rh D-, weak D (Du), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the RHD gene. Blood 1997;89:2568-77.
- Gassner C, Cchmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequencespecific primers. Transfusion 1997;37:1020-6.
- Maaskant-van Wijk PA, Faas BH, de Ruijter JA, et al. Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. Transfusion 1998;38: 1015-21.
- Faas BH, Beckers EA, Wildoer P, et al. Molecular background of VS and weak C expression in blacks. Transfusion 1997; 37:38-44.
- 17. Singleton BK, Green CA, Avent ND, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. Blood 2000;95:12-8.
- Wagner FF, Moulds JM, Tounkara A, Kouriba B, Flegel WA. RHD allele distribution in Africans of Mali. BMC Genet 2003;4:14.
- Wagner FF, Frohmajer A, Flegel WA. RHD positive haplotypes in D negative Europeans. BMC Genet 2001;2:10.
- 20. Okuda H, Kawano M, Iwamoto S, et al. The RHD gene is highly detectable in RhD-negative Japanese donors. J Clin Invest 1997;100:373-9.
- 21. Lee YL, Chiou HL, Hu SN, Wang L. Analysis of RHD genes in

Taiwanese RhD-negative donors by the multiplex PCR method. J Clin Lab Anal 2003;17:80-4.

- 22. Peng CT, Shih MC, Liu TC, et al. Molecular basis for the RhD negative phenotype in Chinese. Int J Mol Med 2003;11:515-21.
- 23. Muller TH, Wagner FF, Trockenbacher A, et al. PCR screening for common weak D types shows different distributions in three Central European populations. Transfusion 2001;41:45-52.
- 24. Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000;95:2699-708.
- 25. Körmöczi GF, Legler TJ, Daniels GL, et al. Molecular and serological characterization of DWI, a novel "high-grade" partial D. Transfusion 2004;44:575-80.
- 26. Flegel WA, Curin-Serbec V, Delamaire M, et al. Section 1B: Rh flow cytometry. Coordinator's report. Rhesus index and antigen density: an analysis of the reproducibility of flow cytometric determination. Transfus Clin Biol 2002;9:33-42.
- Issit PD, Anstee DJ. Applied blood group serology. 4th ed. Miami: Montgomery Scientific Publications; 1998.
- 28. Sachs L. Angewandte statistik. 8th ed. Berlin/Heidelberg: Springer Verlag; 1997.
- 29. Nicolas V, Le Van Kim C, Gane P, et al. Rh-RhAG/ankyrin-R, a new interaction site between the membrane bilayer and the red cell skeleton, is impaired by Rh (null)-associated mutation. J Biol Chem 2003;278:25526-33.
- 30. Daniels G. Human blood groups. 2nd ed. Oxford: Blackwell Science; 2002.
- Perco P, Shao CP, Mayr WR, Panzer S, Legler TJ. Testing for the D zygosity with three different methods revealed altered Rhesus boxes and a new weak D type. Transfusion 2003; 43:335-9.
- 32. Shao CP, Xiong W. A new hybrid RHD-positive, D antigennegative allele. Transfus Med 2004;14:185-6.
- Mollinson PL, Engelfriet CP, Contreras M. Blood Transfusion in Clinical Medicine. 9th ed. Oxford: Blackwell Science; 1993.
- Frohn C, Dumbgen L, Brand, et al. Probability of anti-D development in D- patients receiving D+ RBCs. Transfusion 2003;43:893-8.
- 35. Flegel WA, Khull SR, Wagner FF. Primary anti-D immunization by weak D type 2 RBCs. Transfusion 2000;40:428-34.
- 36. Smith NA, Ala FA, Lee D, et al. A multi-centre trial of monoclonal anti-D in the prevention of Rh-immunisation of RhD- male volunteers by RhD+ red cells. Transfus Med 2000;10(Suppl 1):8.