TECHNICAL PROTOCOL FOR ASSESSMENT OF GENETIC DISTANCE FOR ISF GUIDELINES FOR THE HANDLING OF A DISPUTE ON EDV IN DIPLOID PERENNIAL RYEGRASS

Microsatellite Marker Analysis – Experimental procedures

1. Sampling of leaf material

- Define two random sub-samples of 20 plants / variety for sampling. These two sub-samples are analyzed in bulk. Each bulk is formed by a different set of plants of a given variety.
- Harvest even-sized leaf segments from each plant that contributes to a given bulk. Young leaves should be used for sampling. The twenty leaf segments that constitute a bulk are pooled before further processing**.**

2. DNA extraction of freeze-dried leaf material

- The plant materials can be used directly for DNA extraction or they can be freeze-dried during 48 hours before DNA-extraction. In this latter case, the plant materials should be packed under vacuum conditions to avoid DNA-degradation.
- For DNA-extraction the Nucleon Spin kit (Macherey-Nagel) can be used, following the manufacturer's instructions.
- Check the DNA-quality and DNA-concentration of each DNA-sample either by gel electrophoresis or using a spectrophotometer (e.g. a NanoDrop spectrophotometer, NanoDrop Technologies Inc, Rockland, USA).
- Bring the samples to a standard concentration of 20 ng/µl. DNA-samples with lower values for any of these parameters should be discarded and new DNA-extractions carried out.

3. Fragment analysis

- The PCR-amplification can be carried out either in uniplex of in multiplex PCR-reactions, as similar results are expected.
- In the original work carried out to set-up a suitable procedure, multiplex PCR assays were set up:
  - Five to ten marker loci were amplified simultaneously using primers labelled with different dyes. Publically available microsatellite marker sequences were used ('original denomination' in Table 1). For the set-up of multiplex PCR reactions new primer pairs were developed which amplified the same locus ('new code name' in Table 1).
  - PCR was performed in a 15 µl volume containing approximately 40 ng of template DNA, 1X PCR buffer (components 40mM tricine-KOH, 16mM KCl, 3,75 µg/ml BSA and 3.5mM $MgCl_2$), primer mix: between 0.027 and 0.2 mM of each primer, 0.25 mM of each dNTPs and 0.25 U Titanium*Taq* polymerase. Thermocycling started with denaturation of the template DNA for 2 minutes at 95 °C, followed by 26 cycles of 45 seconds at 95°C, 45 seconds at 58°C, 2 minutes

at 72°C and stopped after a final extension step of 72°C for 6 minutes.

- Samples containing 2 µl PCR reaction and 10 µl Hi-Di formamide were then separated using, a 36 cm capillary array on an Applied Biosystems 3730XL DNA Analyzer. Alleles were detected using GeneScan/Genotyper® software package of Applied Biosystems.

- In principle, other detection systems and suitable scoring software can be used, provided the band separation is carried out with a resolution of at least 1 bp (it should be possible to differentiate PCR-bands that differ in size by 1 bp). The use of a different detection system or the use of uniplex PCR-reactions will require optimization of the PCR-conditions.
- All the DNA-bands amplified in the range 50-500 bp are recorded as 1 or 0, to obtain a vector of values for each fingerprint.

4. Data analysis

- Jaccard similarities are then calculated between pairs of bulks.

5. List of the SSR markers with fragment size range

- In Table 1 the characteristics of the 31 loci used for analysis are summarized. The original and newly developed primer sequences developed to amplify these loci in multiplex PCR reactions are given. Further details can be found in Roldán-Ruiz et al. (in preparation).

Table 1 List of 31 SSR markers with fragment size range, number of identified alleles and allele sizes (based on the sizes determined by ILVO, fragment size might vary based on the used platform and laboratory, the use of reference samples is strongly advised, as the specific sizing of the fragments is system- and laboratory-dependent). The data on number of alleles and range correspond to the results obtained in the study described in Roldán-Ruiz et al. (in preparation)

Original locus denomination	Original primer pair	New code name	New primer pair	# alleles	Range (bp)
B1A2	F: GTGCAGCAGTTTGAATTGGA R: AGCATCGGGAGCTATGAATG	R01	F: TTTGAATTGGATTGGTCTGGTT R: TCGGGAGCTATGAATGATGA	17	116-243
B1A8	F: GACTTTCAGGCATCGGTCAT R: CCCAGCTCCATTCTTAATGC	R02	F: TTGGAAGATGCCAAAGTGAAG R: GGAAGGCTCCATAATTCTCCTC	11	180-222
B1B3	F: AGGTGTCCTGTTGCTTTGGA R: TTTACCCCCAGGGATCAAAT	R03	F: TACAATCCTTGATCCTGTCTGC R: CCCAGGGATCAAATCACATAAA	8	291-403
B1C8	F: TTCTGGCCATGTTGATTTGC R: GTCTACGGGTTGGAGCAGTG	R04	F: GCAGGGTGAATTTGAAGCA R: TACGGGTTGGAGCAGTGG	4	177-184
B1C9	F: GAGCCGATGCACAGGTTACT R: AAAGGAAGCCGGCTAATCAC	R05	F: TCATCGGTCAGGTGTTTCCT R: GCCGGCTAATCACCAAAGT	8	132-168
B2G6b	F: CCAACTAGACAAAGGGGATTG R: GGAGAGCACCATTCATCCAT	R07	F: AGAATTCGGATCACAACCAACT R: GGATCTTGAAGGGCAACG	7	99-129
B3A1	F: CTTGTCGTCCTTGTTGGGAG R: ATATTCTGGATCGTGGCGTT	R08	F: TGCTACTCTTGTCGTCCTTGTT R: CTGGATCGTGGCGTTGTT	3	309-318
B3A3	F: GGGTGAAGTGCTCTTTGTGA R: ATGGTGAAGGCCTGAAACTG	R09	F: GGTAGGGTGAAGTGCTCTTTGT R: CAACCAGTGCTTCAGCTTTGT	4	352-363
B3C10	F: CTACAACTCCGTGCTGCTGA R: TGCATGGTTTCTCAAATGCT	R12	F: GCTGCTGATGCCAACCAT R: GCTCTCTAACACCATATCAACTCG	4	320-323
B3C5	F: TGTCATGTTCAGAAAGTGCG R: TGTCCACATAAATGCACCTCA	R14	F: TGGGAGCACAACATCTCG R: CACATAAATGCACCTCAGAACAA	13	190-213
B3D2	F: ATACGAGCGAATTGCCTCTC R: TCTCCCATCGCTTATGTTCC	R16	F: GGTACCAATAATCAGAGGCTAAACAA R: CGCCACCATTGATGAACTC	9	392-425
B3E6	F: CTGTAACAACAGCCGCTGAG R: GTCTCGAGCACAGGAGTTCA	R18	F: GATCAGCCAACAATCACTCG R: CGCAGCAACAGTCTAACGAT	4	350-354

Original locus denomination	Original primer pair	New code name	New primer pair	# alleles	Range (bp)
B4C4	F: TGCATGCACCCTTGTAGC R: GGAGACTTTGTGTGTGCAGC	R19	F: GGACTCCACCGTGATCCAT R: GATGTTGAGGCTCTCCGACT	10	279-295
B4D7	F: CGGGAGCTCTCTCTCCTTCT R: TCCAGAACCTTCTCGAGGTC	R20	F: TCGCCTCCTCGGTAAGTG R: CCAGCCTTTCCACATACACAA	12	374-402
B5E1	F: AAACATCAACGGAAGGATGC R: TGATATGCATTGTGATGGAGG	R22	F: GCACAAACAGAAATAATCAACACA R: TGATGATGCTTTGCTCTGATG	7	316-329
B5G4	F: TGGAGTTGTGGACCTTTTCC R: AGATGCTGGTTGGTTTCCAG	R23	F: ACATGTTCCAGTGCAAATTAGG R: GAGGCTGATGACGCACAA	7	430-447
LpSSR006	F: CAATGGAGTCCCAACAG R: TACCTGGGCAAATCTTG	R24	F: CATGTGAGGAGGCAAACTTGT R: TTAAAGGATACCTGGGCAAATC	21	235-275
LpSSR011	F: AAATGTTCATCGTATCG R: CAGGTCCCTGCTTAC	R25	F: TGTTCATCGTATCGACATTACAACT R: TGTGTCTCCCTTGGTAGCATT	30	235-290
LpSSR020	F: GGGGAAATACAGTTCTGC R: GATGCTCCTGCCTACTTTA	R26	F: GCACAAACACAATATACACCCTTT R: TACTTTGATGCTCCTGCCTACTTT	19	391-459
LpSSR021	F: AACAAGTCAATGGACAGATT R: TTTGTTTTCCCTTTTGG	R27	F: AACGCATTGTGCATCCATT R: TTGGGTTTGATTTGCAAGAAG	16	158-198
LpSSR023	F: ATGCACGGGTTTTATTCATT R: CGCGAGGCTTAAGGTGT	R28	F: CATTGTTATGGTTGCAAACTTCTT R: TGTTGCATGAGGCGTCTTC	35	229-302
LpSSR057	F: TAGCCTCCAGAAACAAAGTC R: CATAGCAGTACAGCCAGTCA	R30	F: CGTAGCCTCCAGAAACAAAGTC R: TCTTGGGCTTGCATCCAG	12	118-139
LpSSR058	F: CGATGAACTCAAGGGGGATT R: GCACCGGTCTAGGGACAGAA	R31	F: GGTTGGGTGAAATGGGAGAT R: CCGGTCTAGGGACAGAAGTG	17	223-260
LpSSR059	F: GATCGGATCGGTACAGGAGA R: GAAGCGCACCTTCTGTTTCT	R32	F: CGGATCGGTACAGGAGAAAT R: ACAATCGAAGCGCACCTT	17	169-221
LpSSR066	F: GCCAGTGCCCATTCCGATAA R: CCCCACTCCAACCAAAGCAA	R33	F: CTTATGCGCAACAGAGCAAG R: TGCTGTACGTAGTGGCTGATG	33	306-393
LpSSR076	F: CCCATACTTCGAGGCATAAA R: AAATTCCCCCATCAGAGAAC	R34	F: CACGCAACCTGCGATAACT R: GAGTGGAATTTGATGCCTATCATT	18	319-354

Original locus denomination	Original primer pair	New code name	New primer pair	# alleles	Range (bp)
LpSSR085	F: GCCAGATCCCTTGTAGAAG R: GCACCATTTAAAACCAAAGA	R35	F: TTAGATCTGCAATGGATGAGATG R: TGAGGTCCCACCACCAAG	31	108-255
LpSSR091	F: CACTCTCGGTCTCGCCTTAT R: TTCGCATGCATACAACACAT	R37	F: TCCCGATCATCTTGCACA R: CTTCGCATGCATACAACACA	11	116-137
Rye012	F: GGTCTAATTGTCGTCCTTTC R: GAGTGATTTGGAGGTGAGAA	R39	F: GGTTAGCGTTCGGTCGTC R: TCGGGTTACAGAGAGATTGCTT	31	333-455
Rye014	F: CTGCTCTGTGTTTGTGTGAC R: GCCTTTCATCGTTACTGTCT	R40	F: GCTATGCTAGTACCTGCTCTGTGTT R: TCTTGGCTGGAGGCTAGG	23	250-303
Uni001	F: AGCCACACTTTACCTAATGCTG R: CCCGCAAAACTTACAATTAAA	R41	F: AATGCTGCCATGCACGTA R: GCATAAAGACTTACCCGCAAA	14	169-210