Report for course: BI0489 Plant Breeding, Genetic Recourses and Biotechnology at SLU, Alnarp

Assessment of primers for future evaluation of genetic differences in *Lepidium campestre* (L.) by means of PCR based ISSR markers

Juliane Barten
Contact: Juliane.Barten@gmx.de

Abstract

For a domestication project at SLU and within the course „Plant breeding, genetic resources and biotechnology" primers for the upcoming evaluation of genetic differences between *Lepidium campestre* (L.) accessions were tested. ISSR-PCR was used for this purpose. Due to problems during the procedure only eight of ten primers could be tested in the electrophoresis properly. Of these only one resulted in polymorphic band pattern and loci and can be suggested for the future application in the assessment. While two primers need to be tested again this report also suggests assessing further primers that might be suitable evaluation of genetic differences.

Introduction

Within the scope of the course „Plant breeding, genetic resources and biotechnology" at SLU a project was undertaken. While one aim was the application of the imparted knowledge the work was also incorporated in an ongoing research program of the Department of Crop Science, SLU Alnarp.

Assuming that decrease of market prices will lead to a search for alternatives by farmers and taking the high energy demands of currents crops and their influences on leaching of plant nutrients into consideration a long-term program was started at SLU. It aims at domesticating *Lepidium campestre* as a perennial oil crop that could be undersown in a spring cereal. Finally its oil shall be used in the chemical industry. Besides the domestication process a new production system will also be developed. A timetable was done for the years 2004 to 2007 (see "Annex"). The result of the program will be a plant that is ready for cultivation, which is not expected to be available until after 15 to 20 years. (Merker, 2006)

One of the trivial names of *Lepidium campestre* (L.) is wild cress. It is a dicotyl member of Brassicaceae family and native in Europe. There are annual, biennial and perennial forms known. (Tenaglia, 2006)

In order to assess genetic differences in the plant material molecular markers are used in this program. The aim of this single project was to find primers for the ISSR technique. They should provide polymorphic band patterns with markers at loci all over the genome to enable the assessment of genetic differences.

As stated by Hailu et. al. (2005) ISSR is most suited for revealing differences in the genome since this method is faster and cheaper than RFLPs, shows a higher reproducibility than RAPDs and no knowledge of the target sequence is presumed like with SSR. Furthermore it has been used for genetic diversity assessment several times earlier.

picture 1: *Lepidium campestre* (http://pics.davesgarden.com/pics/TuxedoWarwick_1154759920_281.jpg)

The ISSR method uses primers that anneal to inter-repeat sequences on the DNA. Assumed the distance between two inversely repeated microsatelites is sufficient this region will be amplified. (Weising et. al., 2005)

Materials and Methods

Plant Material

Two different accessions of *Lepidium campestre* were sown and grown in the greenhouse in order to produce samples for the project work. The seeds for the plants were obtained in Budapest. The accessions are called 6 and 9 in this work. Two leaf samples were taken from each

plant when the plants reached the four leaf stage. From accession Nr. 6 four samples and from accession Nr. 9 six leaf samples were taken. The different sample number was due to the fact that two samples of another accession from Alrid, Southern Sweden, were already analyzed by Therése Bengtsson (the lab project supervisor) to test the procedure and give examples of proper results.

DNA extraction

After collecting the samples into numbered tubes which were stored on ice, DNA was extracted from the leaves following the given instructions.

In each tube 300 µl of extraction buffer (Tris-HCl (pH 8) (A3454 from Applichem), EDTA (Mw 372.24) (8418 from Merck), NaCl (Mw 58.44) (S0520 from Duchefa), SDS (S1377 from Duchefa)) was given and the material was destroyed with a piston to set free the cell components in the solution. Following 300 µl of phenol/CHCl3/isoamyl (25/24/1, v/v/v, obtained from Sigma-Aldrich, Merck and Applichem) was added and everything mixed. Two minutes centrifuging (Eppendorf Mini-Spin) provided two separated layers in the tubes of which only the upper, aqueous phase was transferred into a new tube. The last two steps were repeated twice. At this point of the procedure the samples 9.4 and 6.2 looked milky compared to the other clear samples. Afterwards 2X volume of ice cold ethanol (Solveco Etanol 99,5% Art nr: 1016 (diluted in water to 99%)) was added and the solution was left alone for 15 minutes before everything was centrifuged at full speed (14.000 rpm). The supernatant was removed as completely as possible and the remaining pellet was washed twice with 500 µl ethanol (Solveco Etanol 99,5% Art nr: 1016 (diluted in water to 75%)) and centrifuged for two minutes. The evaporation of all remaining ethanol followed and 30 µl distilled water (TE-buffer (pH 8)) that contained RNAse (one µl of 20 mg/ml RNAse (Ribonuclease A Type 1-As obtained from Sigma-Aldrich R5503) mixed in 1 ml water (TE-buffer)) was given to the dry pellet. All samples were stored in the fridge.

DNA quality tests

The extracted DNA was tested for quality. Therefore Agarose Gel was prepared: 1.4 % Agarose (type 1; A5093 Sigma Aldrich) was weighed, given into a bottle and 100 ml TBE-buffer (Tris Ultrapure T1000 from Saveen Werner, Boric acid B0503 from Duchefa and EDTA 8418 from Merck) was added. Using a microwave the mixture was boiled in several steps until no Agarose particles could be seen. While the solution cooled down to about 65°C a small electrophoresis tray (Techtum Lab AB; Maxicell) was prepared by putting the boundaries into position on each end of the tray. After adding two drops Ethidiumbromide (40221-10 Ethidium dropper; Mercury) the Agarose was poured in the tray and the combs for the wells were put in after no bubbles could be found.

While the Agarose became solid DNA samples for the test were prepared. Two µl DNA was taken from each probe into a new tube and mixed with six µl H2O and two µl Buffer (6x Loadingbuffer; R0619 Fermentas Life Science (10mM Tris-HCl pH 7.6, 0.03 % Bromophenolblue, 0.03 % Xylene Cyanol, 60 % Glycerol, 60mM EDTA)). All ten samples were centrifuged afterwards.

Then the combs for the wells and the boundaries were removed from the solid Agarose and the TBE-buffer was added until the Agarose was completely covered.

In both first wells a DNA-ladder (GeneRuler 100bp DNA ladder plus SM0321 Fermentas Life Scienc) was put and the upper wells were chosen for the samples of accession Nr. 6 and the others for the samples of accession Nr. 9. While adding the samples to the gel accidentally not enough volume was added to the third upper well (sample Nr. 6.2) which may have influenced the result (see "Results").

The Electrophoresis was then run with 50 V for about 30 minutes. Afterwards a picture of the bubble-free gel was taken under UV-light (Photo-Print IP-215-SD). Since UV-light destroys DNA the photo had to be taken quite fast. The resulting picture should reveal if there were still some contaminations with RNA or if the DNA was pure.

While the gel ran the second quality test was accomplished. The DNA was measured with Nanodrop (ND-1000 Spectrophotometer from Saveen Werner). See in the "Annex" for the exact procedure.

RCR amplification and Electrophoresis

In order to look at the differences between the accessions PCR based ISSR markers were used in this project. One sample of each accession was chosen. Since the four samples of accession Nr. 6 held no promising result in previous tests, one of Therése Bengtsson's samples was taken. The second sample was Nr. 9.5. A total of ten primers were selected for PCR. The primers were called 841, 852, 858, 859, 865, 868, 880, 888, 889 and 890. These primers were provided by Invitrogen (see "Annex" for nucleotide sequences).

The ISSR procedure began with calculating the needed volume of each component. The master mix in table 1 resulted.

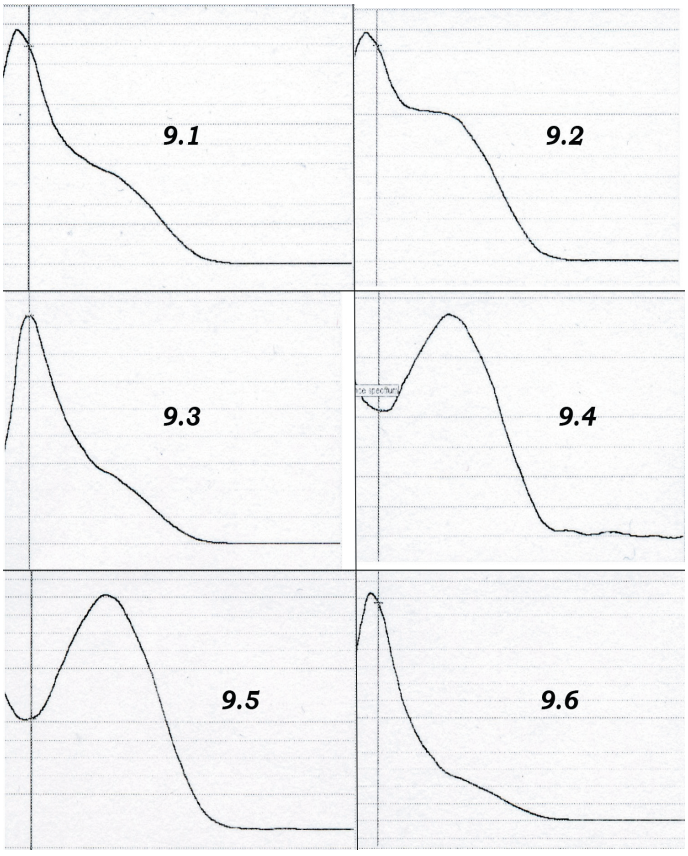
Master mix			
	original concentration	volume/sample	final concentration
Buffer	10x	2.5µl	1x
$MgCl_2$	25mM	2.5µl	2.5mM
dNTPs	25mM	0.2µl	0.2mM
Primer	0.03mM	0.33µl	0.4µM
Taq polymerase	5U/µl	0.1µl	0.02U
H_2O		18.4µl	
DNA	10ng/µl	1.0µl	0.4ng/µl
total volume		25µl	

table 1: Master mix for PCR solution

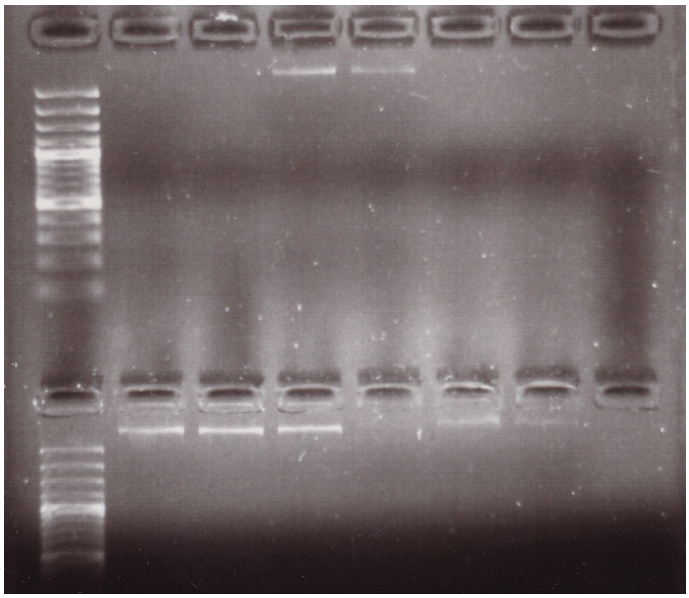
A total of 45 samples were estimated for mixing to be sure to get enough mixture for all 30 samples (10 for each accession and additionally 10 for the water control).

The mixing took place on ice since especially the dNTPs are very escharotic when coming in contact with DNA. First the primer working solution for each primer was produced by taking one µl of primer stock solution in a new tube and diluting it with four µl of sterile water. The rest of the PCR amplification was proceeded in the PCR room. There the dNTP mix was produced after the nucleotides (stored in a freezer) were defrosted. Of each of the four nucleotides (dNTP set 100mM/dNTP. DNS100 Saveen Werner) ten µl were taken and everything was mixed in a new tube. After preparing these two mixtures the rest of the ingredients – starting with the water – were put into the 30 tubes following the given volumes in table 1. In the water control one µl DNA was substituted with one µl sterile water. Finally dNTPs and primers were added and everything was closed with a lid and centrifuged (E-centrifuge Wealtec from Techtum Lab AB) shortly to get rid of air bubbles.

The amplification took place with an ISSR program in the PCR machine (Gene Amp PCR system 9700 from PE Biosystems). The conditions of the program were the following: A hot start at 94°C for one minute followed be 40 cycles of one minute at 94°C, a drop to 55°C at 22% ramp rate for two minutes, rise to 72°C at 55% ramp rate for 30 seconds and then a rise to 94°C of the next cycle at 57% ramp rate. The last cycle was followed by five minutes of product extension at 72°C and a cooling down to 20°C room temperature.



picture 3: Nanodrop curves sample 9 without scale



picture 2: Quality test with Agarose gel

The gel and the TBE-buffer were prepared as described before. This time a bigger tray with 44 wells in total and 250 ml TBE-buffer with 3.75 g Agarose for the gel was used. The amplification product was mixed with four µl loading buffer and 20 µl of mixture was put in each well since the slots turned out to be too small for 29 µl. The GeneRuler 100bp DNA ladder plus SM0321 obtained from Fermentas Life Science was put into the first, eleventh and twenty-second slot of the upper wells and the third and fourteenth slot of the lower wells.

The tube of sample 6.10 (representing primer 890) contained not enough amplification product (see "Discussion") and was therefore not put into the gel.

The gel was then run with 90 V for 2.5 hours and a photo of it was taken under UV-light as explained above.

Results

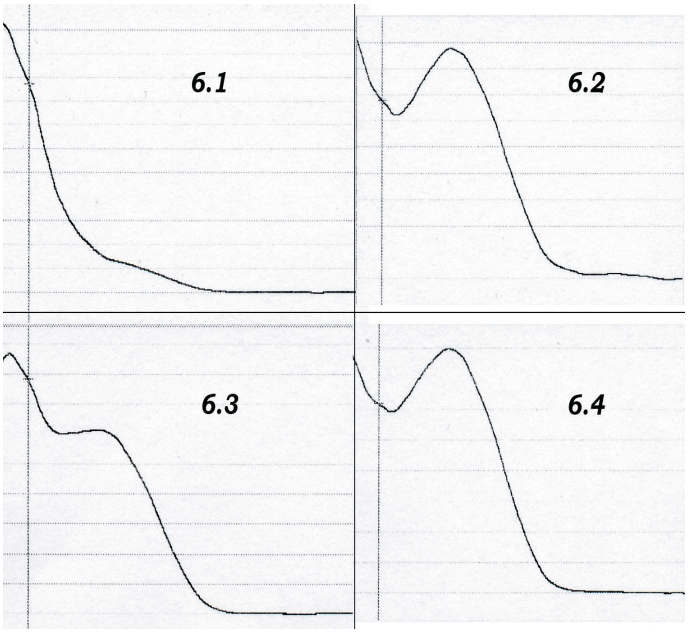
Quality tests

In picture 2 the results of the testing for RNA residues are shown. Since no bands appeared beside the bands close to each well, it can be concluded that there is no contamination with RNA in the samples and the RNAse worked properly.

The quality test with Nanodrop which measured the usable DNA gave very diverse results as shown in picture 3 and 4 and table 2. While sample 6.2 showed no DNA in the Agarose, the Nanodrop measurement showed more present DNA then in sample 6.1 and 9.4 which also provided no band in the first quality test. Nevertheless the mistake while putting the DNA of sample 6.2 into the well seemed not to have influenced the result of both tests greatly.

Concerning table 2 the figure in the column "260/230" should be bigger then 2 and in column "260/280" range between 1.8 and 1.9. Proteins and waste are represented by the figure 230 nm while 280 nm shows aromatic products.

After summing up these results of the quality tests it was decided to investigate only into sample Nr. 9.5 further and use a backup sample for comparison and substitution of accession Nr. 6.



picture 4: Nanodrop curves sample 6 without scale

Sample ID	ng/uL	A260	260/230	260/280	Const.
6.1	33.33	0.667	0.15	2.43	50
6.2	86.78	1.736	1.28	1.97	50
6.3	301.06	6.021	0.77	2.21	50
6.4	197.22	3.944	1.28	2.16	50
9.1	228.02	4.560	0.42	2.21	50
9.2	345.06	6.901	0.68	2.16	50
9.3	264.45	5.289	0.31	2.37	50
9.4 (bubbles)	-4.21	-0.084	0.61	2.63	50
9.4	73.72	1.474	1.74	2.09	50
9.5	163.02	3.260	2.11	2.12	50
9.6	126.68	2.534	0.20	2.41	50

table 2: Nanodrop data

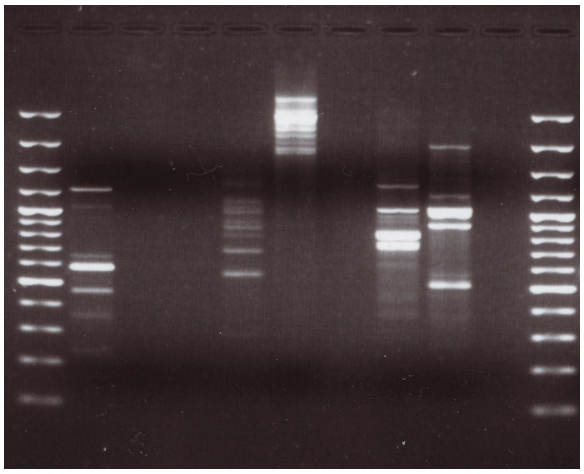
Final results

The finally resulting band pattern of both samples and the water control is shown in pictures 5 to 7.

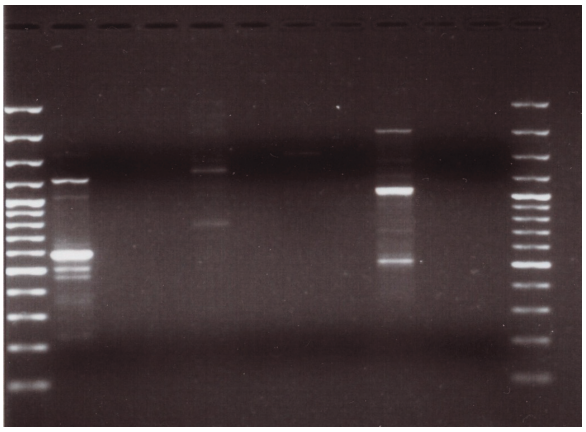
In the water control sample 8 (representing primer 888) showed contamination. Therefore the band pattern of sample 6.8 and 9.8 could not be taken into consideration although they show some small differences. Also sample 10 could not be investigated since there was no enough amplification product for all three slots. Four primers produced no band pattern at all. These primers were 852, 858, 868 and 889 and tested in the slots number 2, 3, 6 and 9. For accession number 6 primer 865 and 880 (slot 5 and 7) gave good bands but no bands occurred in the opposite sample number 9. Primer 859 resulted in a weak band pattern for both accessions, while primer 841 in the first slot produced strong bands.

Concerning the polymorphism of bands it can be stated in general that the first primer gave (except for one loci) no polymorphic band pattern and primer number 859 produced for both accessions almost completely different patterns.

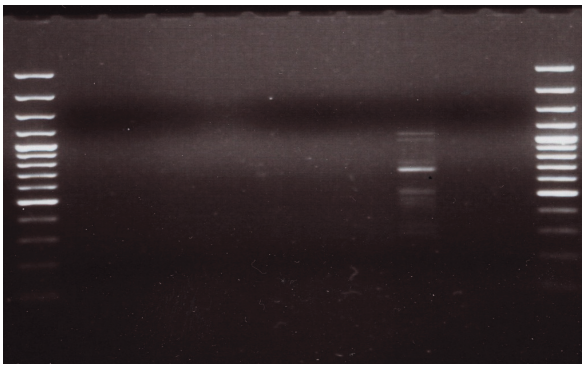
More bands appeared in accession Nr. 6 than in Nr. 9. The loci within the bands patterns were scored as “present” or “absent” and also quite weak loci were included as “present” to increase the number of loci and regard the circumstances of the production of the results (see “Discussion”). A range of zero to nine loci per primer was produced. When not taking the contaminated sample and sample 10 into consideration a total of 40 loci were produced by eight primers. Concerning the two primers that gave band patterns for both accessions a total of 26 loci could be counted of which ten (38.46%) where polymorphic. For primer 841 only one polymorphic loci could be found while primer 859 produced nine polymorphic loci.



picture 5: Band pattern sample Nr. 6



picture 6: Band pattern sample Nr. 9



picture 7: Band pattern water control

Discussion

The work on this project and the results showed that these procedures are very sensible and susceptible for mistakes, especially when working in the lab for the first time. Therefore the results should be looked at very carefully.

Regarding the results of the quality tests first problems occurred after analyzing the figures produced by the Nanodrop measurement. While the samples showed no contamination with RNA, other ingredients that were not wanted could be measured. To guarantee reliable results after PCR amplification and electrophoresis another sample from the backup was used.

The reasons for the contamination and also for the milky appearance of two samples during the DNA extraction are most likely to be found in the performance of the work that was done by unprofessional.

The next problem occurred after the PCR amplification when sample Nr. 6.10 did not contain enough product to work with further. Since this was not the case before starting the program it can be concluded that the lid was not closed sufficient and therefore the content could evaporate. Through this mistake primer Nr. 890 was not tested in the electrophoresis.

For evaluation of the band patterns and loci also weak bands were included as "present" in order to regard the circumstances of the production of the results where maybe the purity and the amount of DNA was lower then usual. As stated in "Results" only two primers allowed conclusions concerning genetic differences. This is especially too few when recognizing that one of these primers produced a band pattern that was only different in one loci. Primer 888 and 890 need to be tested again for their band patterns since the first could give no reliable results due to contamination and the last was not tested as stated before. All other primer excluding primer 859 should not be used further since they allowed either no comparisons due to missing band patterns in one accession or in case of primer 841 the results were not polymorphic enough.

In conclusion it can be stated that more testing of primers is necessary to be finally able to assess genetic differences. From this work only one primer can be advised for further usage. Primer 859 gave good polymorphic band pattern even though the loci appeared to be weak in the Agarose gel.

Annex

Timetable of four years of the research program

2004

Interspecific hybridisation
Induction of mutations
Investigation of further F1 to F3 and isolation of recombinants
Trials with undersowing
Transformation experiments
Studies of oil content and quality

2005

Investigation of further F1 to F4 from interspecific hybrids
Growing of M1 from X-ray treatment
New and old trials with undersowing
Transformation experiments
Studies on oil content and quality

2006

Studies of F2 to F5 from interspecific crosses and further isolation of recombinants
Growing of large M2 populations and isolation of mutants
Transformation experiments
Isolation of shatterproof genes
Evaluation of undersown Lepidium from 2005

2007

Further growing and evaluation of materials from crosses and mutants
Trials with new interesting lines
Isolation of shatterproof genes and attempts to transform them into L. campestre
Increases of new materials

Start the printer (red button)

Press ND-1000 icon on the desktop

Press Nucleic Acid

Wipe the nanodrop with Kleenex

Add 1-2 µl sterile H_2O

Press OK to initialize the instrument

When initialized choose DNA-50 in the upper right corner

Wipe the nanodrop with Kleenex

Add 1-2 µl sterile H_2O and press BLANK

When blanked wipe the nanodrop with Kleenex again

Add 1-2 µl of your first DNA sample

Write the name of your sample in the box

Press Measure

When measured clean with Kleenex again and add the new sample

Repeat until you have measured all of your samples

When you have finished all samples clean the machine with sterile water and wipe with Kleenex

Press Print Report

Press Exit

picture 8: Quality testing procedure with Nanodrop

Applied Primers

The Primers used in this project were part of the UBC Primer set ordered from Invitrogen. The nucleotide sequences are like following:

841
GAG AGA GAG AGA GAG AYC
852
TCT CTC TCT CTC TCT CRA
858
TGT GTG TGT GTG TGT GRT
859
TGT GTG TGT GTG TGT GRC
865
CCG CCG CCG CCG CCG CCG
868
GAA GAA GAA GAA GAA GAA
880
GGA GAG GAG AGG AGA
888
BDB CAC ACA CAC ACA CA
889
DBD ACA CAC ACA CAC AC
890
VHV GTG TGT GTG TGT GT

Acknowledgment

I am grateful for the tutorial in the lab, advice and help from Therése Bengtsson and Arnulf Merker. Furthermore I want to thank SLU for providing the facilities for the work that would not have been possible in this complexity at my home university.

References

Hailu, Faris; Merker, Arnulf; Belay, Getachew; Johansson, Eva; 2005; *Molecular diversity and polygenic relationships of tetraploid wheat species as revealed by inter-simple-sequence repeats (ISSR) from Ethopia*

Merker, Arnulf; 05.12.2006; pers. Comm.

Tenaglia, Dan; 08.12.2006; www.missouriplants.com/Whitealt/Lepidium_campestre_page.html,

Weising, Kurt; Nybom, Hilde; Wolff, Kirsten; Kahl, Günter; 2005; *DNA Fingerprinting in Plants – Principles, Methods, and Applications*; second edition; Boca Raton