http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (12): 2303-2307, 2006
ISSN 1028-8880

Comparison of AFLP Polymorphism in Progeny Derived from Dichogamous and Homogamous Walnut Genotypes

Mehmet Sutyemez
Department of Horticulture, Faculty of Agriculture, University of Kahramanmaras Sutcuimam, Kahramanmaras-Turkey

Abstract: In walnut, genetic variability between progenies of homogamous and dichogamous genotypes is important especially when they are used as rootstock seed source. In this study, level of polymorphism and genetic diversity between progeny and maternal tree of dichogamous ('Sutyemez-1') and homogamous ('Maras-18') walnut genotypes were studied using Amplified Fragment Length Polymorphism (AFLP) technique. DNAs of ten progenies from each maternal tree were compared with the maternal one using four AFLP primer combinations. Totally 102 bands were amplified and 28.4% of them were polymorphic with an average of 25.5 total and 7.2 polymorphic bands per primer pair in dichogamous 'Sutyemez-1' genotype. In homogamous 'Maras-18' genotype, 99 fragments were amplified and 27.3% of them were polymorphic with an average of 24.7 total bands and 6.7 polymorphic bands per primer pair. Slightly higher number of bands and polymorphism in the dichogamous genotype was attributed to the out-crossing of the genotype. The average number of total character difference between maternal tree and progeny in homogamous genotype (13.1) was found higher than the dichogamous genotype (10.5). This was explained by having higher level of heterozygosity present in homogamous genotype.

Key words: AFLP, diversity, *J. regia*, polymorphism, walnut

INTRODUCTION

J. regia is the most common cultivated *Juglans* species in Europe and Turkey is one of the origins of *J. regia* and is fourth walnut producer country after ABD, Iran and China in the world with 125 thousand tons nuts per year (FAO, 2003).

Walnut trees are monoecious and wind is pollinating agent. *J. regia* is heterodichogamaous species i.e., having dichogamous and homogamous cultivars. Dichogamous includes protandrous and protogynous cultivars and causes out-crossing. In dichogamy, male and female flowers bloom at different periods in the spring. Male inflorescences flower earlier than female ones in protandry, whereas female flowers bloom earlier than male ones in protogeny. Dichogamy may sometimes create problems in the orchard during pollination by bearing blank nuts and low nut set. Therefore, it is suggested to establish new walnut orchards with two cultivars of which flowering periods overlap (Sen, 1986). The genetic variability between progenies of homogamous and dichogamous genotypes is important especially when they are used as rootstock seed source. In Turkey, there is no specific seed source in the rootstock production for walnut cultivation and variable seed sources are used for the propagation of grafted walnut plants by the nurserymen. In this point, knowing level of genetic variability between the progenies as well as between maternal tree and progeny is critical.

DNA-based markers provide useful information in fingerprinting and assessing genetic diversity between accessions. Of the various kinds of DNA-based markers characterized so far, amplified fragment length polymorphism technique (AFLP; Vos *et al*., 1995) appears to be most promising for varietal fingerprinting, parent identification and genetic diversity studies. One of the main advantages of AFLP technique is its multiplex ratio, which means that a larger number of amplified products are generated in a single reaction (Powell *et al*., 1996).

In walnut, there is only one study using AFLP technique by Kafkas *et al*. (2005) which determines genetic relationships between walnut genotypes originated from Turkey. RFLP, RAPD and ISSR molecular marker techniques were also applied to *J. regia* to determine existence of apomixis, to establish phylogenetic relationships in the genus *Juglans*, to assess genetic diversity and to characterize cultivars, to find marker linked to hyper-sensivity to the cherry leafroll virus, to identify inter-specific hybrids and to construct genetic

linkage map in the previous studies (Aly *et al*., 1992; Fjellstrom *et al*., 1994; Fjellstrom and Parfitt, 1994a, b; 1995; Woeste *et al*., 1996; Malvolti *et al*., 1997; 2001; Nicese *et al*., 1998; Potter *et al*., 2002).

Although, there are few molecular studies in walnut, none of them compared level of polymorphism and genetic diversity within dichogamous and homogamous genotypes at the DNA level. In this study, we aim to detect level of polymorphism and genetic variability between maternal tree and its progeny in a dichogamous and in a homogamous walnut genotype by AFLP analysis.

MATERIALS AND METHODS

Plant material: Progenies of 'Sutyemez-1' (dichogamous-protogynous) and 'Maras-18' (homogamous; syn. Kalayci) obtained from open pollination were used in this study. To get seedlings, the nuts were stratified +4°C for two months and were germinated, then transplanted to the plastic bags in the greenhouse. The leaves of ten progenies from each genotype and of maternal trees were collected for DNA isolation and for molecular fingerprinting.

DNA extraction: The collected leaf samples were frozen in liquid nitrogen and stored at -70°C until use after washing and drying. Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle (1987) with minor modifications. The leaves (1 g) were ground in liquid nitrogen and put into the 15 mL tubes and mixed with 6 mL of CTAB buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 2% PVP, 0.2% β-mercaptoethanol, 0.1% $Na_2S_2O_5$). Samples were incubated at 65°C for 1 h with gentle shaking every 5-10 min, mixed with an equal volume of chloroform-isoamyl alcohol (24: 1) for 15 min, with occasional gentle shaking every three min and centrifuged at 5.500 rpm for 15 min. The chloroform-isoamyl alcohol step was repeated two times. The aqueous phase was recovered and mixed with an equal volume of cold isopropanol and left at -70°C for 2 h. The precipitated nucleic acids were either recovered by centrifugation at 1,000 rpm for 3 min or transferred into 1.5 mL eppendorf tube by needle or pipette tip and then washed with 10 mM ammonium acetate in 76% ethanol, the pellets were dried and resuspended in dd H_2O. Concentration of extracted DNA was estimated by comparing band intensity with λ DNA of known concentrations, after 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA was diluted to 25 ng μL^{-1} for PCR reactions.

Table 1: Sequences of oligonucleotide adaptors and primers used in AFLP analysis

Adaptor/primer	Code	Sequence
Adaptors		
*Eco*RI adaptors		5'- CTC GTA GAC TGC GTA CC -3'
		3'- CAT CTG ACG CAT GGT TAA -5'
*Mse*I adaptors		5'- GAC GAT GAG TCC TGA G -3'
		3'- TA CTC AGG ACT CAT -5'
Preselective amplification primers		
*Eco*RI primer + A	E_A	5'- GACTGCGTACCAATTC+A-3'
*Mse*I primer +C	M_C	5'- GATGAGTCCTGAGTAA+C-3'
Selective amplification primers		
*Eco*RI + 3-ACG	E_{ACG}	5'- GACTGCGTACCAATTC+ACG-3'
*Mse*I + 3-AGA	M_{AGA}	5'- GATGAGTCCTGAGTAA+AGA-3'
*Mse*I + 3-AGC	M_{AGC}	5'- GATGAGTCCTGAGTAA+AGC-3'
*Mse*I + 3-AGT	M_{AGT}	5'- GATGAGTCCTGAGTAA+AGT-3'
*Mse*I + 3-ATA	M_{ATA}	5'- GATGAGTCCTGAGTAA+ATA-3'

AFLP reactions: For AFLP analysis, 250 ng of genomic DNA from each sample was digested with 5 units of *Eco*RI and 5 units of *MseI* in a final volume of 25 μL. The reaction buffer was 10X RL-buffer (100 mM Tris. Hac pH 7.5, 100 mM MgAc, 500 mM KAc, 50 mM DTT). This mixture was incubated for 2 h at 37°C. To the double digested DNA sample, a total volume of 5 μL of ligation reaction mixture including 5 pM of *Eco*RI adapter and 50 pmoles of *MseI* adaptor, 10 mM ATP, 1 unit of T4-DNA ligase and the same reaction buffer as the above mentioned were added and incubated for 5 h at 37°C.

The pre-selective amplification reaction mixture contained 2.5 μL restricted-ligated DNA as PCR template, 50 ng μL^{-1} of each of the pre-selective amplification primers (*Eco*RI+A and *MseI*+C), 2 mM dNTPs, PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$ and 1 units of *Taq* polymerase (MBI Fermentas Inc., Vilnius, Lithuania). The reaction volume was 25 μL. PCRs were performed as described by Vos *et al*. (1995) with minor modifications: 20 cycles of 30 sec at 92°C, 30 sec at 60°C and 60 sec at 72°C. After pre-amplification, the PCR products were diluted 1:20 with dd H_2O. The adaptor sequences, pre-selective amplification primers and selective primers are listed in Table 1.

The labeling reaction contained 5 ng of *Eco*RI adaptor binding selective primers, 0.05 units T4 DNA kinase, 1 X T4 DNA kinase buffer, 0.05 μL of [λ ^{33}P]- ATP (3000 Ci/m mol) in a final volume of 0.25 μL per selective amplification PCR. The labeling reaction was performed for 40 set of selective-amplification PCRs at 37°C for 1 h and the enzyme was inactivated by 10 min of incubation at 70°C.

The selective amplification reaction was conducted in a final volume of 10 μL containing 2.5 μL of diluted pre-selective amplification product as a template, 3 ng labelled *Eco*RI site primer, 15 ng *Mse*I site primer, 2 mM dNTPs, PCR buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$ and 1 unit of *Taq* polymerase. The cycling conditions were 11 cycles of denaturation at 94°C for 30 sec, annealing at 65°C (0.7°C/each cycle) for 30 sec,

extension at 72°C for 60 sec and additional 24 cycles of denaturation, annealing and extension at 94°C for 30 sec, 56°C for 30 sec and 72°C for 60 sec, respectively. A total of 10 μL of the AFLP selective amplification product was mixed with 10 μL of loading buffer (98% formamide, 10 mM EDTA, 0.25% each of bromophenol blue and xylene cyanol FF), then denatured at 94°C for 5 min and placed immediately on ice. Electrophoresis was performed on an EC160 standard sequencing unit (Thermo Electron Corporation, Milfort, USA). Two PCR reactions were performed for each primer combinations.

About 3 μL of mixture were loaded onto a 4.5% (w/v) polyacrylamide denaturing gel with 0.5 X TBE buffer after a pre-run electrophoresis at 60 V for 30 min and then were run at 60 V until the loading dye reached to the bottom of the gel. The gels were dried at 80°C for 3 h. Hyperfilm-MP (Amersham, England) was exposed to the gels for 2 day.

Band scoring and data analysis: The AFLP bands were scored as present (1) or absent (0). Only the clearest and strongest bands were scored and evaluated for the analysis. Total band (AFLP band) differences between maternal genotype and progeny were calculated. Genetic distances between all pair-wise combinations of genotypes were calculated by using PAUP package (Swofford, 1998). The distance values are based on the proportion of different bands between all the possible pairs of genotypes.

RESULTS AND DISCUSSION

Level of polymorphism: AFLP analysis resulted with adequate polymorphism between the maternal genotypes ('Sutyemez-1' and 'Maras-18') and their progenies. AFLP banding patterns of the walnut genotypes and their progenies using E_{ACG}/M_{AGT} primer combination are shown in Fig. 1.

In dichogamous 'Sutyemez-1' genotype, we had totally 102 bands and 29 of them (28.4%) were polymorphic with an average of 25.5 total and 7.2 polymorphic bands per primer pair (Table 2). In homogamous 'Maras-18' genotype, four primer combinations amplified totally 99 fragments and 27 of them (27.3%) were polymorphic with an average of 24.7 total bands and 6.7 polymorphic bands per primer pair (Table 3). So, the level of polymorphism and the number of total bands were slightly higher in the dichogamous genotype than the homogamous one as expected. In 'Sutyemez-1' genotype, male and female flowers overlap only one or sometimes two days, whereas in 'Maras-18' genotype they overlap at least 5-7 days (Sutyemez, 1998). These data shows therefore effect of out-crossing on the level polymorphism.

Kafkas *et al.* (2005) characterized 21 Turkish walnut cultivars by AFLP analysis. The authors used same primer pairs that we used in this study and they found about 50% polymorphism among walnut cultivars. This means

Table 2: Total number of bands, number of polymorphic bands and percentage of polymorphism detected in dichogamous 'Sutyemez-1' genotype

Primer combinations	Total No. of bands	No. of polymorphic bands	% of polymorphism
E_{ACG}/M_{AGA}	23	5	21.7
E_{ACG}/M_{AGC}	21	6	28.6
E_{ACG}/M_{AGT}	24	7	29.2
E_{ACG}/M_{ATA}	34	11	32.3
Total/Average	102/25.5	29/7.2	28.4

Table 3: Total number of bands, number of polymorphic bands and percentage of polymorphism detected in homogamous 'Maras-18' genotype

Primer combinations	Total No. of bands	No. of polymorphic bands	% of polymorphism
E_{ACG}/M_{AGA}	23	6	26.1
E_{ACG}/M_{AGC}	20	6	30.0
E_{ACG}/M_{AGT}	22	4	18.1
E_{ACG}/M_{ATA}	34	11	32.3
Total/Average	99/24.7	27/6.7	27.3

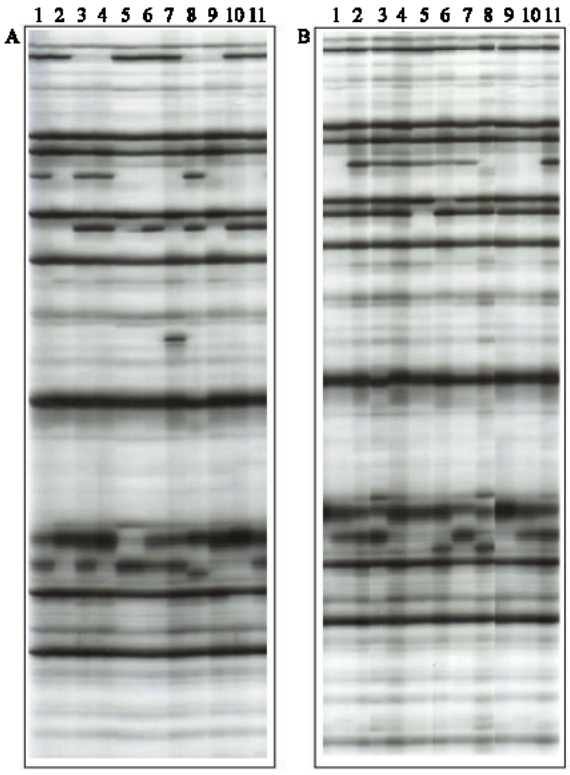

Fig. 1: AFLP banding patterns of the walnut genotypes and their progenies using E_{ACG}/M_{AGT} primer combination (A) Dichogamous 'Sutyemez-1' genotype, (B) Homogamous 'Maras-18' genotype. Lanes: 1. Maternal genotype, 1-11. Progenies

Table 4: Numbers and percentages of total band differences between maternal genotypes and their progeny

	Sutyemez-1		Maras-18	
	No.	(%)	No.	(%)
Progeny-1	13	12.7	15	15.2
Progeny-2	11	10.8	18	18.2
Progeny-3	8	7.8	16	16.2
Progeny-4	8	7.8	10	10.1
Progeny-5	11	10.8	19	19.2
Progeny-6	9	8.8	10	10.1
Progeny-7	11	10.8	11	11.1
Progeny-8	12	11.8	8	8.1
Progeny-9	13	12.7	16	16.2
Progeny-10	9	8.8	8	8.1
Total/Average	105/10.5	10.2	131/13.1	13.2

Table 5: Pairwise genetic distances among walnut genotypes calculated by PAUP program. Above diagonal represents 'Sutyemez-1' and its progenies, while below diagonal exhibits 'Maras-18' and its progenies

No.	Genotypes	1	2	3	4	5	6	7	8	9	10	11
1	Maternal	-	0.127	0.107	0.078	0.078	0.107	0.088	0.107	0.117	0.127	0.088
2	Progeny-1	0.151	-	0.156	0.127	0.107	0.098	0.098	0.156	0.107	0.137	0.137
3	Progeny-2	0.181	0.050	-	0.107	0.147	0.137	0.156	0.058	0.127	0.156	0.078
4	Progeny-3	0.161	0.050	0.040	-	0.137	0.127	0.147	0.088	0.137	0.147	0.088
5	Progeny-4	0.101	0.151	0.141	0.181	-	0.068	0.068	0.147	0.098	0.127	0.127
6	Progeny-5	0.191	0.141	0.111	0.111	0.131	-	0.098	0.137	0.088	0.117	0.098
7	Progeny-6	0.101	0.171	0.161	0.141	0.101	0.111	-	0.137	0.127	0.098	0.137
8	Progeny-7	0.111	0.121	0.111	0.090	0.151	0.101	0.070	-	0.147	0.156	0.117
9	Progeny-8	0.080	0.171	0.161	0.181	0.040	0.131	0.080	0.131	-	0.088	0.068
10	Progeny-9	0.161	0.050	0.060	0.080	0.101	0.111	0.121	0.131	0.121	-	0.117
11	Progeny-10	0.080	0.131	0.121	0.101	0.101	0.111	0.060	0.090	0.080	0.141	-

that the level of polymorphism between walnut cultivars is almost 100% higher than the one between maternal tree and progeny that revealed 25% polymorphism.

The percentage of polymorphism obtained from this study is similar with RAPD analysis of 19 walnut cultivars (Nicese *et al*., 1998). However, average number of polymorphic bands per primer pairs (6.7-7.2) in this study was about 5.2 and 1.7 times more than RAPD (Nicese *et al*., 1998) and ISSR (Potter *et al*., 2002) studies, respectively which characterized walnut cultivars originated from different countries, Average number of polymorphic bands per primer between *J. regia* and *J. nigra* by RAPD analysis was reported as 2.6 by Woeste *et al*. (1996) which is 2.6 times lower than our study. These data show usefulness and effectiveness of AFLP technique in the characterization of very closely walnut accessions.

The maximum number of bands (34) and the highest level of polymorphism (32.3%) were obtained from primer combination E_{ACG}/M_{ATA}, whereas the least number of bands was generated with primer pair E_{ACG}/M_{AGC} in both genotypes (Table 2 and 3) that agree with Kafkas *et al*. (2005). The lowest level of polymorphism was generated with primer combination E_{ACG}/M_{AGA} (21.7%) in 'Sutyemez-1' genotype and E_{ACG}/M_{AGT} (18.1%) in 'Maras-18' genotype.

Genetic diversity between progenies and maternal genotypes: Total band differences between dichogamous 'Sutyemez-1' genotype and its progenies changed between 8 and 13, whereas it ranged from 8 to 19 in homogamous 'Maras-18' genotype (Table 4). The percentage of total band differences between 'Sutyemez-1' and its progenies changed between 7.8 and 12.7%, whereas it ranged from 8.1 to 19.2% in 'Maras-18'. The average number of total band difference was 10.5 in 'Sutyemez-1' and it was 13.1 in 'Maras-18', whereas the average percentages were 10.2 in 'Sutyemez-1' and 13.2 in 'Maras-18'.

'Sutyemez-1' maternal genotype had smaller genetic distance to its progenies 4, 5, 7, 11 than the others (Table 5). In 'Maras-18' genotype, progenies 5, 7, 8, 9, 11 had smaller genetic distances values than the other progenies. The maternal genotype 'Maras-18' had approximately 0.132 unit genetic distance, whereas 'Sutyemez' maternal genotype had 0.102 unit.

So, homogamous 'Maras-18' genotype had more number and percentage of total band difference and smaller genetic distance values to its progenies than protogynous 'Sutyemez-1' genotype. This may be explained by existence of higher level of heterozygosity in 'Maras-18' genotype as well as diversity of different pollen source. Since, the sampling was performed in the walnut germplasm and there were a few adjacent walnut genotypes overlapping with 'Maras-18'. Number of overlapping days for 'Maras-18' was six in the year that seed was formed for seedlings used in this study.

Total band differences between progenies ranged from 6 to 16 in 'Sutyemez-1', whereas it was between 4 and 18 in 'Maras-18'. The percentage of total character

differences between progenies of 'Sutyemez-1' ranged from 9.6 to 13.0%, whereas it was between 10.1 and 12.4% in 'Maras-18' (data not shown).

As a result, this study reveals that AFLP technique can be used successfully in walnut to discriminate very closely walnut genotypes, to determine parents of a genotype, in inheritance and linkage analysis in walnut.

ACKNOWLEDGMENTS

I am grateful to Dr. Salih Kafkas and Dr. Hakan Ozkan for their comments on the manuscript and molecular analysis.

REFERENCES

Aly, M.A.M., R.G. Fjellstrom, G.H. Mcgranahan and D.E. Parfitt, 1992. Origin of walnut somatic embryos determined by RFLP and isozyme analysis. HortScience, 27: 61-63.

Doyle, J.J. and J.L. Doyle, 1987. A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., 19: 11-15.

FAO., 2003. FAO Web Page. (http://apps.fao.org/faostat).

Fjellstrom, R.G., D.E. Parfitt and G.H. Mcgranahan, 1994. Genetic relationships and characterization of Persian walnut (*J. regia* L.) cultivars using restriction fragment length polymorphisms (RFLPs). J. Am. Soc. Hortic. Sci., 119: 833-839.

Fjellstrom, R.G. and D.E. Parfitt, 1994a. Walnut (*Juglans* sp.) genetic diversity determined by restriction fragment length polymorphisms. Genome, 37: 690-700.

Fjellstrom, R.G. and D.E. Parfitt, 1994b. RFLP inheritance and linkage in walnut. Theor. Applied Genet., 89: 665-670.

Fjellstrom, R.G. and D.E. Parfitt, 1995. Phylogenetic analysis and evolution of genus *Juglans* (Juglandaceae) as determined from nuclear genome RFLPs. Plant Sys. Evol., 197: 19-32.

Kafkas, S., H. Ozkan and M. Sutyemez, 2005. DNA polymorphism and assessment of genetic relationships in walnut (*Juglans regia* L.) genotypes based on AFLP and SAMPL markers. J. Am. Soc. Hortic. Sci., (In Press).

Malvolti, M.E., M. Spada, I. Beritognolo and F. Cannata, 1997. Differentiation of walnut hybrids (*Juglans nigra* L. x *Juglans regia* L.) through RAPD markers. Acta Hortic., 462: 43-52.

Malvolti, M.E., B. Fornari, E. Maccaglia and F. Cannata, 2001. Genetic linkage mapping in an intraspesific cross of walnut (*Juglans regia* L.) using molecular markers. Acta Hortic., 544: 179-185.

Nicese, F.P., J.I. Hormaza and G.H. Mcgranahan, 1998. Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers. Euphytica, 101: 199-206.

Potter, D., F. Gao, G. Aiello, C. Leslie and G.H. Mcgranahan, 2002. Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars. J. Am. Soc. Hort. Sci., 127: 75-81.

Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski, 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed, 2: 225-38.

Sen, S.M., 1986. Ceviz yetiştiriciliği. Eser Matbaası, Samsun, 229 s.

Sutyemez, M., 1998. Kahramanmaras bolgesinde ceviz (*Juglans regia* L) seleksiyonu ve secilmis bazi tiplerin dollenme biyolojileri uzerine arastirmalar. Ph.D Thesis, University of Cukurova, Adana-Turkey.

Vos, P., L. Hogers, M. Bleeker, T. Van De Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau, 1995. AFLP: A new technique for DNA fingerprinting. Nucl. Acids Res., 23: 4407-4414.

Woeste, K., G.H. Mcgranahan and R. Bernatzky, 1996. Randomly amplified polymorphic DNA loci from a walnut backcross [(*Juglans hindsii* × *Juglans regia)* × *Juglans regia*]. J. Am. Soc. Hortic. Sci., 121: 358- 361.