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Phylogenetic relationships among *Triticum* L. and *Aegilops* L. species based on the internal transcribed spacer sequences of nrDNA (ITS)

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□ ABSTRACT □

The research Studies genetic relationships among *Triticum* L. and *Aegilops* L. species by direct sequencing of PCR-amplified internal transcribed spacer (ITS) of nuclear ribosomal DNA to investigate the polymorphism in nucleotide sequences among 8 *Aegilops* L. and 7 *Triticum* L. species. ITS sequences were aligned with CLUSTAL W 2.1 multiple sequence aligment program. The phylogenetic relationships among species were reconstructed using Unweighted Pair Group Mean Arithmetic Average (UPGMA) and neighbor-joining (NJ) methods. ITS region ranged from 600 to 602 bp. The length of ITS1 was 221-222 bp, and ITS2 was 215-217 bp. The 5.8S subunit was 163 bp long. The G + C content of the ITS1 region ranged from (61.2 to 63.9)% in all *Triticum* and *Aegilops* species. The G + C content of the 5.8S subunit was entirely identical in all species, 59.5%. The G + C content of the ITS2 region ranged from (59.9 to 63.5)%. There were 54 variable sites (8.97%) in the entire ITS region. *T. dicoccon, T. durum* were more variable than other species.

The phylogenetic relationships among species were reconstructed using (UPGMA) and discussed. There were mainly three clades in this tree. *T. urartu,* was separate from *T. monococcum*. The similarity between *T. dicoccoides* and *T. monococcum* could be the result of a recent introgression event.

Keywords: *Triticum*, *Aegilops*, genetic relationships, internal transcribed spacer, ITS.

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العلاقات التطورية بين أنواع من *Aegilops* L. و*Triticum* L. باستخدام internal transcribed spacer sequences(ITS) nrDNA

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□ ملخّص □

يهدف هذا البحث الى دراسة علاقات القرابة الوراثية بين أنواع من *Aegilops* L. و *Triticum* L. باستخدام internal transcribed spacer (ITS) وقد استخدم في هذه الدراسة 8 أنواع من *Aegilops* L. و 7 أنواع من *Triticum* L. تم تحليل التسلسل لمنطقة (ITS) باستخدام برنامج (CLUSTAL W 2.1 multiple sequence aligment program) ودرست علاقات القرابة الوراثية بين الأنواع باستخدام المتوسط الحسابي للمجموعات الزوجية غير المزانة (Unweighted Pair Group Mean Arithmetic Average (UPGMA و . (NJ)and neighbor-joining

تراوح طول منطقة (ITS) الكلي بين 600-602 bp, طول منطقة ITS1 (221 – 222) bp, طول منطقة ITS2 (215– 217) bp، وطول منطقة 5.8S (163) bp. أما النسبة المئوية للمحتوى من السيتوزين والغوانين (G + C) في المناطق الثلاث السابقة فكانت {(61.2 – 63.9) –(59.9– 63.5)–(59.5)}% على التوالي. أظهرت النتائج وجود 54 موقع متغير في منطقة (ITS) بنسبة مئوية (8.97%). تميز كلا من *T. dicoccon* و *T. durum* بالمواقع الأكثر تغيراً مقارنة مع الأنواع الاخرى المدروسة.

وتم مناقشة و رسم مخطط القرابة الوراثية بين الأنواع المدروسة بطريقة (UPGMA). أظهرت شجرة القرابة الوراثية وجود 3 مجموعات. وبعد النوعين *T. urartu* و*T. monococcum* عن بعضهما. بالاضافة الى التشابه الوراثي بين *T. dicoccoides* و*T. monococcum* العائد الى الفترة القريبة لحدوث التهجين بينهما.

الكلمات المفتاحية : *Aegilops*،*Triticum* ، العلاقات الوراثية، internal transcribed spacer، ITS.

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1.Introduction:

In the last decade numerous molecular markers and techniques were used for studies on origin, evolution and relationships in the wheat group: chloroplast and nuclear microsatellite markers (Lelley *et al.,* 2000; Ishii *et al.,* 2001), chromosome-specific low-copy DNA (Liu *et al.,* 2003), nuclear genes (Caldwell *et al.,* 2004) and ITS has also been used to study the evolution of wheat species in the early 1980s (Peacock *et al.,* 1981; Dvorák and Appels, 1982; Wang *et al.,* 2000; Rudnóy *et al.,* 2005). The evolution of the ITS region is more complicated in hybrid and polyploid species (Baldwin *et al.,* 1995; Wendel *et al.,* 1995; Waters and Schaal, 1996). Baldwin *et al*., (1995) proposed that ITS sequences would provide direct evidence of reticulate evolution if concerted evolution failed to homogenize the repeat units contributed by different parental species when the hybridization event was recent, or if nrDNA repeats were at different loci in the parental genomes and interlocus gene conversion was inoperative in their hybrid, or if the hybrid was asexual. Since the history of polyploid wheats is relatively short (Mori *et al.,* 1995) and the ITS repeats in polyploid wheats are located at different loci (Dubkovsky and Dvorák, 1995; Badaeva *et al.,* 1996), it may be possible to identify ITS sequences of different parental origins in polyploid wheats and hence to identify their progenitors. In addition, the *Triticum* complex is a good model system for studying how hybridization and polyploidization could possibly affect the evolution of nrDNA (Zhang *et al.,* 2002). Numerous studies have demonstrated the utility of the ITS region (Hsiao *et al.,* 1995a; Wang *et al.*, 2000; Blattner, 2004; Jakob *et al*., 2010) for resolving relationships among closely related species in *Triticeae* and other plant species (Hsiao *et al.,* 1994, 1995a,b; Baldwin *et al.,* 1995; Goel *et al.,* 2002; Sharma *et al.,* 2002; Zhang *et al.,* 2002; Alvarez and Wendel, 2003; Bordbar *et al.,* 2011). Hsiao et *al.,* (1995a) studied the sequence of the ITS region of 30 diploid *Triticeae* species representing 19 genomes. they suggest that the sequence of the ITS is variable enough to differentiate closely related species (Hsiao et *al.,* 1994). Carvalho *et al*., (2011) In their present study, 51 durum wheat cultivars showed 40% of ITS variation, and this lower level of polymorphism could be due to the absence of the D genome. Low ITS variation was previously reported for *Triticeae* (Zhang *et al*., 2002) and other taxa such as *Cucurbitaceae* (Jobst *et al*., 1998); *Oleaceae* (Jeandroz *et al*., 1997), and *Vigna* (Saini *et al*., 2008). So Carvalho *et al*., (2011) concluded that the knowledge of the genetic relationships and phylogenies among the durum wheat cultivars and their botanical varieties might contribute for the designing of intraspecific crosses between the genotypes studied there, with potential interest for wheat improvement .

The *Aegilops* genus includes the wild relatives of cultivated wheat. It can play an important role in broadening the cultivated wheat genepool, and thus shows a potential interest for utilization in wheat improvement through introgression of their genes. The present study employed molecular marker technology, to detected genetic relationships among *Triticum* and *Aegilops* species at the DNA level.

2. Materials and methods

2.1. Plant materials: The plant material consisted of 15 accessions (Tabel1). 8 accessions representing (8) *Aegilops* L species: *Ae. tauschii, Ae. speltoides, Ae. ventricosa, Ae. searsii, Ae.cylindrica, Ae. longissima, Ae. bicornis, Ae. sharonensis.* and(7) accessions representing 7 *Triticum* L. species: *T. monococcum, T. urartu, T. dicoccoides, T. durum, T. turgidum, T. dicoccon, T. aestivum* (Table1). All accessions were obtained from the Genetic Resources Unit (GRU) at the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria.

2.2. DNA extraction:

Total genomic DNA was isolated from fresh, young leaves as described by (Doyle and Doyle, 1987). DNA was quantified using Spectrometer and the concentration of all samples was set at 10 ng/ µl.

2.3. PCR amplification:

PCR reactions were carried out in a 25 µl volume containing 10× PCR buffer (Eurobio), dNTPs (10 mM) (Mix Roche), 10×MgCl2 (50 mM) (Eurobio), Taq polymerase (5 U/µl) (Eurobio). ITS region (including ITS1, 5.8S and ITS2) was amplified using the following primer pair (White *et al.,* 1990): ITS-4(5'-TCCTCCGCTTATTGATATGC-3')-ITS-5(5'GGAAGTAAAAGTCGTAACAAGG-3'), DNA was added to each PCR at a rate of 10 ng and the total volume was adjusted with dd H2O to 25 µl. For 35 cycles, PCRs were subjected to 95°C for 1min for DNA denaturation, 50°C for 1 min for annealing of primers, 72°C for 1 min for extension of the target region and 72°C for 5 min for final extension. PCR products (1–5µl) were digested according to manufacturer (Fermentaz). Digested fragments were separated by electrophoresis on 1,8% agarose gel that was run at 100 V for 2 h in TBE 0,5x buffer and visualised under UV lights (Fig 1).

2.4. Sequence analyses:

The PCR products delivered to Leibniz University Hannover-Department of Plant Biotechnology-Hannover-Germany for sequencing, which was conducted in both forward and reverse directions using the amplification primers. ITS sequences were aligned with CLUSTAL W 2.1 multiple sequence aligment program. The boundaries of the ITS region was determined by comparison with the sequence information in (Chatterton *et al*., 1992). The phylogenetic relationships among species were reconstructed using Unweighted Pair Group Mean Arithmetic Average (UPGMA) and neighbor-joining (NJ) methods.

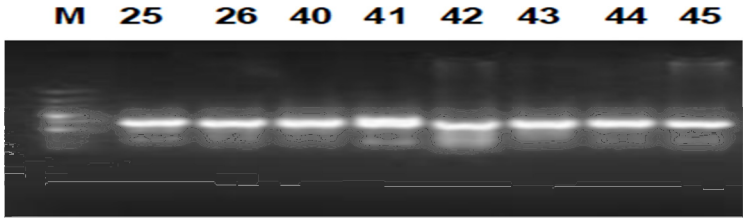

Fig. 1. PCR of ITS in *Aegilops* species. M=100 bp DNA ladder;
25= *Ae. bicornis*; 26= *Ae. sharonensis*; 40= *Ae. speltoides*; 41= *T. turgidum*; 42= *T. dicoccon*; 43= *T. aestivum*; 44= *Ae. ventricosa*; 45= *Ae. cylindrica*.

3.Results and Discussion:

Earlier studies in the *Poaceae* have focused on morphology, anatomy, taxonomy, physiology, cytology, genetics and crop improvement. They have provided important information, but data based on these studies are not enough to assess the true relationships between these species. Phylogenetic constructions proposed for the *Triticum* and *Aegilops* species based on these characters are poorly resolved and differ widely in topology. Therefore we used (ITS) regions of the nuclear ribosomal DNA (nrDNA) because it has been shown to be a valuable source of evidence to resolve phylogenetic relationships in many angiosperm groups (Gulbitti-Onarici *et al*., 2009).

The results of this study showed that all *Triticum* L. and *Aegilops* L. species presented a 700-bp PCR product of invariant length (Fig1). And The ITS region ranged from 600 to 602 bp. The length of ITS1 was 221-222 bp, and ITS2 was 215-217 bp. The

5.8S subunit was 163 bp long (Table1). The total length of the entire ITS of rDNA among *Triticum* L. and *Aegilops* L. species during the present study were variable and were in agreement with the results of earlier studies.

Our results showed that there is a 1-bp indel in ITS1 sequences of *T. turgidum* at position 211 and an 2-bp indel in ITS2 sequences of *T. monococcum* at position (510-511).

Based on the results of this study The G + C content of the ITS1 region ranged from (61.2 to 63.9)% in all *Triticum* and *Aegilops* species. The G + C content of the 5.8S subunit was entirely identical in all species, 59.5%. The G + C content of the ITS2 region ranged from (59.9 to 63.5)% (Table1).

Our present study showed that There were 54 variable sites (8.97%) in the entire ITS region. The ITS1 region had 22 variable sites and the ITS2 region had 32 variable sites. In the 5.8s gene there were no variable sites. And that 16 of 54 variable sites were in *T. dicoccon*, *T. durum*.

In the current study, among the substitutions, transitions were more frequent than transversions, the transitions in ITS1 were 17 while in ITS2 were 25. the transversions in ITS1were 4 while in ITS2 were 5. And among the indels, deletions were one in ITS1 and 2 in ITS2 (Table 2).

Table 1. Base compositions of ITSl, 5.8S, and ITS2 regions of *Triticum* and *Aegilops* species

Triticum and *Aegilops* species		ITS1	%CG	5.8	%CG	ITS2	%CG	ITS Total	%CG
Ae. bicornis	25_R_primer-5R	222	61.7	163	59.5	217	63.1	602	61.4
Ae. sharonensis	26_R_primer-5R	222	61.7	163	59.5	217	63.1	602	61.4
Ae. searsii	33_R_primer-5R	222	61.5	163	59.5	217	63.1	602	61.3
Ae. longissima	34_R_primer-5R	222	62.5	163	59.5	217	63.1	602	61.7
Ae. tauschii	35_R_primer-5R	222	61.2	163	59.5	217	60.3	602	60.3
Ae. speltoides	40_R_Rev-Primer	222	61.5	163	59.5	217	63.5	602	61.5
T. turgidum	41_R_Rev-Primer	221	61.5	163	59.5	217	60.8	601	60.6
T. dicoccon	42_R_Rev-Primer	222	61.2	163	59.5	217	63.1	602	61.2
T. aestivum	43_R_Rev-Primer	222	61.2	163	59.5	217	60.8	602	60.6
Ae. ventricosa	44_R_Rev-Primer	222	62.6	163	59.5	217	59.9	602	60.6
Ae. cylindrica	45_R_Rev-Primer	222	63.9	163	59.5	217	60.3	602	61.2
T. monococcum	48_R_Rev-Primer	222	61.2	163	59.5	215	61.3	600	60.6
T. urartu	25_Rev_5R-Rev	222	61.2	163	59.5	217	61.2	602	60.6
T. dicoccoides	26_Rev_5R-Rev	222	61.5	163	59.5	217	60.6	602	60.5
T. durum	27_Rev_5R-Rev	222	61.5	163	59.5	217	60.6	602	60.5

Table 2. the gray bases indicate transition, transversion, deletion

T. dicoccon	TCTATTTAAT
T. durum	GCTATTTAAT
T. turgidum	-CTATAAAAT

A characteristic conserved sequence GGCRY-(4to7n) GYGYCAAGGAA (where Y=C or T, R=G or A), was also available in the ITS1 of both *Triticum* L. and *Aegilops* L. species (Fig2). In previous studies on many flowering plants this characterstic sequence has been reported in the middle of ITS1 and this sequence is presumed as a recognition site

for processing of a primary transcript into the structural rRNA (Liu and Schardl, 1994). So we can notice this conserved sequence in the middle of ITS1 at position (134 -154) (GGCGCCGAAGGCGTCAAGGAA) and we observe 2 transversions (G A) at position 140 in two *Aegilops* species *Ae. bicornis, Ae. searsii*.

TCGTGACCCT GACCAAAACA GACCGCGCAC GCGTCATCCA ATCCGTCGGC
GACGGCATCG TCCGTCGCTC GGCCAATGCC TCGACCACCT CCCCTCCTCG
GAGCGGGTGG GGGCTCGGGG TAAAAGAACC CAC**GGCGCCA AAGGCGTCAA**
GGAACACTGT GCCTAACCCG GGGGCATGGC TAGCTTGCTA GCCGTCCCTC
GTGTTGCAAA GCTATTTAAT CCACACGACT CTCGGCAACG GATATCTCGG
CTCTCGCATC GATGAAGAAC GTAGCGAAAT GCGATACCTG GTGTGAATTG
CAGAATCCCG CGAACCATCG AGTCTTTGAA CGCAAGTTGC GCCCGAGGCC
ACTCGGCCGA GGGCACGCCT GCCTGGGCGT CACGCCAAAA CACGCTCCCA
ACCACCCTCA TCGGGAATCG GGATGCGGCA TCTGGTCCCT CGTCTCGCAA
GGGGCGGTGG ACCGAAGATC GGGCTGCCGG TGTACCGCGC CGGACACAGC
GCATGGTGGG CGTCCTCGCT TTATCAACGC AGTGCATCCG ACGCGCAGCC
GGCATTATGG CCTCAGAATG ACCCAGCAAA CGAAGCGCAT GTCGCTTCGA
CC

Fig. 2. The ITS sequence of *Ae. bicornis* The highly conserved area within the ITS1 is indicated in bold

The phylogenetic tree of 15 *Triticum* and *Aegilops* species generated using the Unweighted Pair Group Mean Arithmetic Average (UPGMA) and neighbor-joining (NJ) methods was shown in (Fig3). Two trees are essentially identical. There were mainly three clades in this tree. The first clade included *T. dicoccoides, T. monococcum, Ae. searsii, Ae. longissima*. The second clade consisted of *T. turgidum, T. aestivum, T. dicoccon, T. durum*. The third clade contained of *Ae. speltoides, Ae. bicornis, Ae. sharonensis, Ae. tauschii, Ae. cylindrica, Ae. ventricosa*, *T. urartu* (Fig3).

T. urartu, T. monococcum belong to the *Einkorn* wheat group (Mizumoto *et al.,* 2002). These two species proposed as the A genome doner to polyploid wheats (Dvorák *et al.,* 1993; Takumi *et al.,*1993). The results of this study showed that *T. urartu,* was separate from *T. monococcum* this is also supported by previous studies such as those based on analysis of ethanol soluble protiens (Konarev, 1980) and the phylogenetic tree of Vakhitov *et al*., (2003) based on sequences of promoter of rDNA.

Our results revealed that Two different types of ITS sequences were found in *Triticum* tetraploid species. One type (*T. dicoccoides*) formed a group with *T. monococcum*. And the other type (*T. turgidum*) consisted of a group with other *Triticum* species. This finding is supported by Zhang *et al*., (2002) mentioned that the A genome of *T. dicoccoides* was originated from *T. monococcum* based on the ITS2 sequences of nuclear ribosomal DNA. The similarity between *T. dicoccoides* and *T. monococcum* could be the result of a recent introgression event (Fig3).

Tsunewaki and Ogihara, (1983) noted that S^b and S' plasma types found in *sitopsis* section showed much closer relation to A plasma type of *Einkorn* wheat than to other plasma types (B, G and S) of the same section. More over Wang *et al*., (1997) based on two trees (illustrates the phylogenetic trees constructed by unweighted pair-group method using arithmetic averages (UPGMA) and neighbor-joining (NJ) methods) showed that *Einkorn* is closer to *Aegilops* than to *Triticum.* A similar result was reported by (Cenkci *et al*., 2008). So our results supported these proposed ones that, *T. monococcum* had a closer relationships with *Ae. searsii, Ae. Longissima.*

Wheats (*Triticum* spp.) form a polyploid series with diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) forms. The diploid wheats comprise a single

genomic group with the genome formula AA (*T. monococcum, T. urartu*). The tetraploid emmer wheats are divided into two groups, those with the genome formula AABB (*T. turgidum*) and those with the genome formula AAGG (*T. timopheevi*). On evidence it appears that the wild allotetraploid emmer wheat *T. turgidum* ssp. *dicoccoides* (AABB) arose by amphyploidy between the wild diploid wheat *T. urartu* (AA) and a diploid member of the *Aegilops* genus (BB) (Rudnóy *et al.,* 2002). The origin of B genome is still a matter of debate. Polyphyletic origin or divergent evolution of B genome from the donor species are hypothesized. On the basis of chondriome divergence *Ae. speltoides* seems to be the cytoplasm donor (female parent) of the tetraploid wheats (Wang *et al*., 2000). A descendant of *T. turgidum* ssp. *dicocoides*, the *T. turgidum* ssp. *dicoccon* was probably the ancient tetraploid from which hexaploid wheats (AABBDD) may have evolved by hybridisation between the AABB tetraploid as cytoplasm donor and the D genome diploid *Ae. taushii*. (Huang *et al*., 2002). Our results in the second clade reflect these facts and close relationships between tetra and hexaploid wheats are supported by our findings based on ITS analysis and clustered together (Fig3). Inaddition *Triticum* species were grouped in one cluster within *Aegilops* species.

The current study showed that *Ae. speltoides* was separated from remaining four *Sitopsis* species and formed a sister group with *Ae. tauschii*. Zhang *et al*., (2002) reported that Phylogenetic analysis demonstrated that *Ae. speltoides* was distinct from other species in *Aegilops* sect. *Sitopsis*. And the relationships among *Ae. tauschii*, *Ae. speltoides* and subsect. *Emarginata* were not well-resolved. Also Sliai and Amer, (2011) revealed that *Ae. speltoides* does not form a monophyletic clade with other *Sitopsis* species (Goriunova *et al*.,2008; salina *et al* ., 2006). Recent studies showed that *Ae. speltoides* was the main contributor of the B genome of polyploid wheats (Huang *et al.,* 2002). Inaddition the sequence of one chloroplast gene (*rbcL*, for the Rubisco large subunit) from seven *Triticum* and *Aegilops* species indicated that *Ae. speltoides* is the donor of both the plasmon and B genome of common wheat (Terachi *et al.,* 1988; Wang *et al.,* 1997; Gupta *et al.,* 2008; Al-ahmar *et al*., 2010). And the tree reconstructed based on data of ten EST-SSRs mapped on the B genome showed that *Ae. speltoides* had the closest relationship with *T. aestivum* and *T. durum* (Zhang *et al*., 2002). Yen *et al*., (2005) observed the cytoplasm of *T. turgidum* L. is very similar to that in some races of *Ae. speltoides*.

In our ITS analysis, a close relationship of the sequences from *Ae. Tauschii* (*Ae. squarrosa*) and *Ae. cylindrica* was found. Wang *et al*., (1997) reported that *Ae. squarrosa* is the maternal parent of three tetraploids, *Ae. cylindrica*, *Ae. crassa*, and *Ae. ventricosa* (Wan *et al*., 2002). Also according to Queen *et al*., (2004) The three D genome-containing *Aegilops* species, comprising the two members of Section *Vertebrata* (*Ae. ventricosa, Ae. tauschii*; van Slageren 1994) and *Ae. cylindrica* form a clade. More over Kharazian, (2008) said *Ae. taushii* and *Ae. cylindrica* have similar genome grouped together in the application of Rf data (the migration distance of the band/distance of solvent front) (Jaaska, 1981, 1993) but in the MW (the molecular weight of prolamin bands) are separated. Wang *et al*., (2000) provided an approach to understand the genome evolution of allopolyploid species in the genus *Aegilops* through studying the Evolution of parental ITS regions of nuclear rDNA in allopolyploid *Aegilops* (*Poaceae*) species. And they assumed that they might be largely homogenized by concerted evolution toward one of their other ancestors during the process of hybridization and polyploidization. These evidences have been observed in this study. Based on this conclusion we observed a close relationships between (*Ae. tauschii* and *Ae. cylindrica*).

In the third clade there was a sister group (*Ae. sharonensis, Ae. bicornis*) (Fig3). Mendlinger and Zohary, (1995) reported that *Ae. sharonensis* was found to be equally close to *Ae. bicornis*.

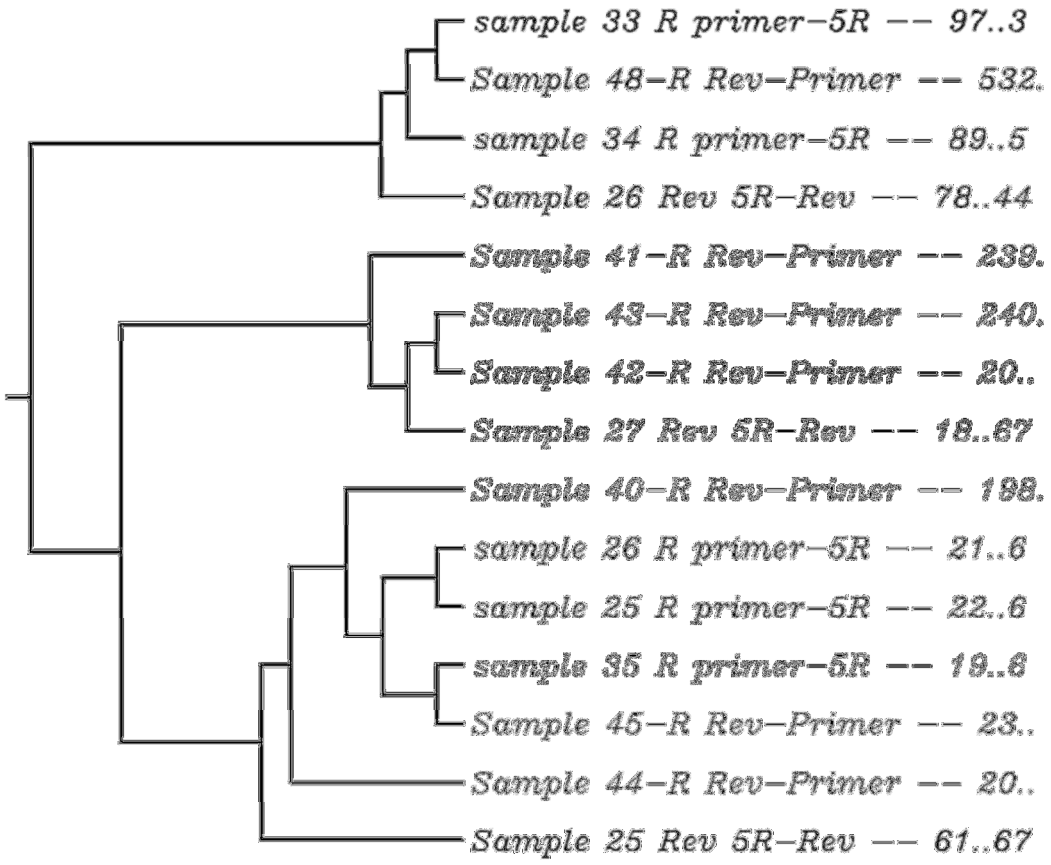

Fig. 3. Phenogram of UPGMA cluster analysis in *Triticum* L. and *Aegilops* L. species

4. conclusions and recommendations:

1- ITS2 was more variable than ITS1.

2- *T. dicoccon, T. durum* were more variable than other species.

3- The similarity between *T. dicoccoides* and *T. monococcum* could be the result of a recent introgression event.

4- Conserved area was identical in the ITS1 of both *Triticum* and *Aegilops* species except two *Aegilops* species *Ae. bicornis, Ae. searsii*.

5- There were 54 variable sites in ITS region.

6- Transitions were more frequent than transversions and the deletions were one in ITS1 and 2 in ITS2.

7- We can identify ITS sequences of different parental origins in *Triticum* and *Aegilops* species and hence identify their progenitors.

8- ITS uesd for resolving relationships among closely related species in *Triticeae* and other plant species.

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