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Research Article

Molecular phylogeny based on its sequences of nrDNA of some species belonging to dodder (*Cuscuta* L.) genus from various ecological sites of Turkey

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Abstract

Nuclear ribosomal DNA (nrDNA) sequence data of the *Cuscuta* genus, which have been considered as one of the most popular sequences for phylogenetic inference in plants, have been studied from a phylogenetic perspective in agricultural and non-agricultural lands of Turkey. The samples of *Cuscuta* spp. were collected from different geographical regions of Turkey between the years of 2013-2015. Some other species, not available locally, were taken from the herbarium samples of some research units. In order to study the phylogenetic relations of collected species, DNA isolations were made from body tissue samples. Conserved regions on ribosomal DNA (rDNA) were amplified by universal primers via PCR method and cloned into a proper cloning vector. The cloned DNA fragments were sequenced and analysed by web-based and computer programs. DNA sequences of certain species were recorded to the National Center for Biotechnology Information (NCBI) database. Based on the morphological examination and molecular analyses of fresh and the herbarium specimen, 8 species were identified. The identified species were *C. hyalina* (Gene bank accession no. KY020420), *C. monogyna* (KY020421), *C. europaea* (KY020422), *C. palaestina* (KY020423), *C. approximata* (KY020424), *C. kurdica* (KY020427), *C. kotschyana* (KY020430) and *C. babylonica* (KY020431). The ITS (Internal Transcribed Spacer) region contains several indels in identified *Cuscuta* species with the length varying from 668 to 730 bp. Sequence divergence ranges from 1.00% to 8.00% within *Cuscuta* spp. Based on our findings, the ITS sequences provided phylogenetically informative results in combination with the secondary structures.

Keywords: classification; *Cuscuta* spp.; molecular phylogeny; Turkey

Introduction

Dodder (*Cuscuta* spp.) is evaluated as a flowering parasitic plant in the family of Convolvulaceae according to some researchers (Yuncker, 1932; Kujit, 1969; Parker and Riches, 1993; Linao *et al.*, 2000). However, some others have been evaluating in the family of Cuscutaceae (Hadac and Chrtek, 1970; Davis,

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1978; Chertek and Obsornova, 1991). About 200 species are belonging to the genus *Cuscuta* and are commonly regarded as noxious weeds or invasive plants. *Cuscuta* is the third most harmful parasite plant group in the world after *Striga* and *Orobanche* (Parker and Riches, 1993; Costea and Tardif, 2006; Costea *et al.*, 2006; Garcia *et al.*, 2014). Dodder, *Cuscuta* species, is a parasitic annual plant that infests many agricultural crops such as alfalfa, clover, beans, soy, cranberry, some members of the grass family, trees, herbaceous plants, shrubs and various ornamentals in worldwide (Yunker, 1932; Li, 1987; Dawson *et al.*, 1994; Maberley, 1997; Stefanovic and Olmstead, 2004). Members of the genus were recently implicated as vectors in the horizontal transfer of mitochondrial genes in plants (Mower *et al.,* 2004).

Dodders are lack of vegetative organs such as root or leaf. The diagnosis of these plants can only be done by observation of flower and fruit characters. In general, difficulties are encountered in diagnosis made with taxonomic characters. Therefore, the identification of molecular phylogenetic relationships in the diagnosis of these plants provides a more reliable diagnosis (Nemli *et al.*, 2015).

Cuscuta is an annual plant producing seeds that germinate on or near the soil surface. Germination is influenced by temperature, with an optimum at 30 °C (Benvenuti *et al.*, 2005). *Cuscuta* spp. can produce 3.000-25.000 seeds. Most of the seeds remain dormant for 5-15 years, close to the soil surface (Hutchisan and Ashton, 1979).

Yuncker (1932) has been studied the *Cuscuta* through systematic studies about evolutionary relationships, character evolution, biogeography, and speciation. Stefanovic *et al.* (2007) has been defined as the major lineages of the subgenus Grammica, which is the largest and the most complicated infrageneric taxon of *Cuscuta*. Costea *et al.* (2008) have examined the phylogeny-based classification of the genus *Cuscuta*.

New methods of phylogenetic analysis have made it possible to clarify the early evolution of Magnoliophyta (Podoplelova and Ryzhakov, 2005). Until 1960, systematic information was obtained based on morphological and behavioral variations. After the 1960s, biological macromolecules have played an important role in evolutionary and systematic studies (Soltis *et al.*, 1997).

PCR amplification of the ITS region on ribosomal DNA is now a popular option for phylogenetic analysis of closely related species and populations. This popularity has been enhanced by the production of universal primers that can bind to conserved rDNA regions. The data obtained as an analysis of ITS base sequences provide significant contributions to the solution of existing problems in the taxonomic categories. The relationships of the concerned taxa are usually determined by studying the ITS variations of the taxa. The major non-coding nuclear DNA region has been widely used for reconstruction, identification, and phylogenetic analysis of many plant families. ITS has been used in countless systematic studies at a wide variety of plant species and species levels (Baldwin *et al.*, 1995).

Keskin *et al.* (2017) have been reported the analysis of nrDNA's ITS sequences as an efficient tool to study the phylogeny of dodders collected from various provinces of Eastern Anatolia (Turkey). The sequences of 18S rRNA, ITS-1, 5.8S rRNA, ITS-2, and 26S rRNA regions of 4 *Cuscuta* species were determined by molecular cloning and sequencing. This study results clearly showed that the most stable secondary structure derived from the sequences obtained by universal ITS4 and ITS5 primers is a very efficient tool for the identification of *Cuscuta* species when used in combination with phylogenetic analysis.

Today, the universal primers are used for amplification and sequencing of the ITS region. Primers were originally designed for fungal rRNA amplification and are derived from fungal (*Saccharomyces*), insect (*Drosophila*), and plant (*Oryza sativa* and *Hordeum vulgaris*) sequences (White *et al.*, 1990). Phylogenetic trees based on the sequence analysis results of ITS-1 or ITS-2 regions may produce results not supported by other sequences. Therefore, the results of combining the information obtained from the ITS-1 and ITS-2 regions reveal more accurate, robust, and complete trees (Baldwin *et al.*, 1995; Baldwin, 1992). This study aims to determine the phylogenetic relations of species belonging to the genus *Cuscuta* in Turkey.

Materials and Methods

Taxon sampling

The plant materials of *Cuscuta* spp. were either field-collected from different geographical regions of Turkey or provided by herbarium samples of some universities between the years of 2013 and 2015 (Figure 1), as indicated in the Flora of Turkey (Davis, 1978). A list of the 8 species of *Cuscuta* included in the analysis with their voucher deposition data, gene bank accession numbers, sources, collection site, and the length of amplified sequences are provided in Table 1.

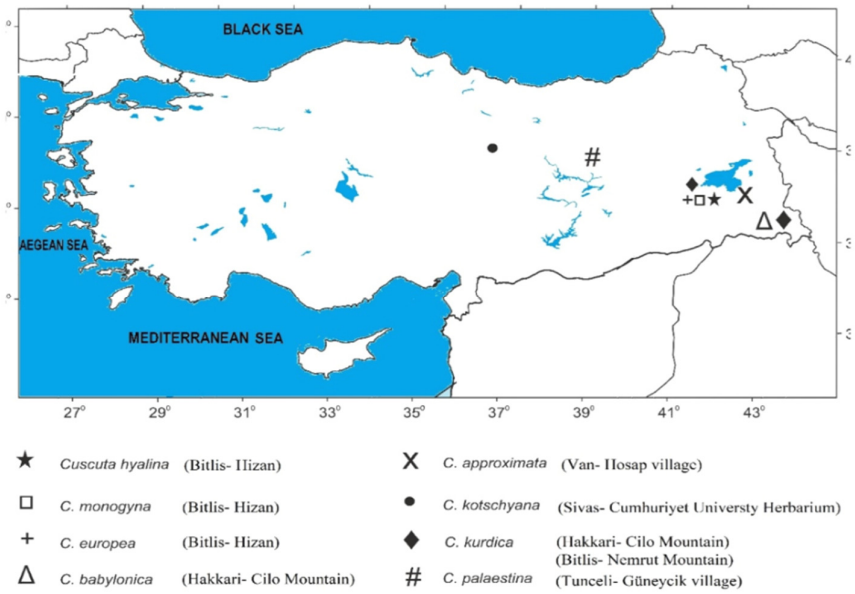


Figure 1. Locations and the species of *Cuscuta* spp. collected from different geographical regions of Turkey

Table 1. EMBL accession number and the source of sequences used

Taxa	No of Sample and locality	Gen Bank accession number	Length (bp)	NCBI database
Cuscuta hyalina	1769 (Bitlis-Hizan)	KY020420	674	HM748873.1
Cuscuta hyalina	Canada	HM748873	621	
Cuscuta hyalina	Turkey	KX683007	674	
Cuscuta monogyna	1770 (Bitlis-Hizan)	KY020421	677	AY554405.1
Cuscuta monogyna	UK	AY554405	737	
Cuscuta monogyna	China	KY456203		
Cuscuta monogyna	Spain	DQ924569	606	
Cuscuta monogyna	Armenia	MH109269	636	
Cuscuta kotschyana	1817 (Cumhuriyet Univ. Her.)	KY020430	682	DQ924587.1
Cuscuta kotschyana	Iran	DQ924585	610	
Cuscuta kotschyana		DQ924587	611	
Cuscuta europaea	1785 (Bitlis-Hizan)	KY020422	730	AY554401.1
Cuscuta europaea	UK	AY554401	739	
Cuscuta palaestina	1792 (Tunceli Güneycik)	KY020423	682	AY554406.1
Cuscuta palaestina	UK	AY554406	721	
Cuscuta approximata	1808 (Van-Hoşap)			

Cuscuta approximata	1809 (Van-Hoşap)	KY020424	682	KU725873.1
Cuscuta epithymum	1810 (İzmir-Kemalpaşa)	KY020425	682	KU725873.1
Cuscuta kurdica	1811 (Bitlis-Nemrut Mountain)	KY020426	685	KU725873.1
Cuscuta kurdica	1811 (Hakkari-Cilo Mountain)	KY020427	678	DQ924613.1
Cuscuta kurdica	Turkey	KY020428	679	DQ924613.1
Cuscuta babylonica	1819 (Hakkari-Cilo Mountain)	DQ924613	607	
Cuscuta babylonica	Turkey	KY020431	611	DQ924578.1
Cuscuta babylonica	Afganistan	DQ924579	696	
Cuscuta babylonica		DQ924578	596	

DNA isolation and PCR: amplification

Genomic DNA was extracted either from fresh *Cuscuta* spp.

Plants collected from the fields or attempted using dried material from herbarium specimens using a commercial DNA preparation kit (Thermo Scientific, USA). Species identification of collected plant samples was conducted basically on molecular (sequence analysis and secondary structure pattern of nrDNA) and morphological (flower structure) analyses.

The major non-coding nuclear DNA region (ITS1, 5.8S, and ITS2) were amplified by polymerase chain reaction (PCR) with universal oligonucleotide primers (ITS4-5'-TCCTCCGCTTATTGATATGC-3' and ITS5-5'-GGAAGTAAAAGTCGTAACAAGG-3') of White *et al.* (1990). The amplification of the ITS1/5.8S/ITS2 region was performed by polymerase chain reaction (PCR) in a final volume of 50 µl. The PCR mixture contained: 2 µl of genomic DNA, 5 µl of 10× reaction buffer (200 mMTris-HCl pH: 8.4, 500 mMKCl), 3 µl of MgCl2 (25 mM), 1 µl of dNTPs (10 mM each), 1 µl of each primer (100 pmol), 0.4 µl of DNA polymerase, and 36.6 µl of DNase free sterile water. The thermal regime of PCR reaction was set according to White *et al.* (1990). PCR products were separated and detected in a 1.5% agarose gel as described by Sambrook (1989). The DNA fragments were isolated from the gel using Isolate II PCR and Gel Kit (Bioline, Germany). Purified DNA fragments were cloned and sequenced in both directions using two universal (SP6 and T7) primers by Iontek Company (Turkey). The accession numbers of the sequences studied in this paper have been given in Table 1 and deposited in the GenBank database.

Molecular cloning and sequencing

A total of 8 ITS sequences including ITS1/5.8S/ITS2 regions were molecularly cloned and sequenced. The upstream sense primer ITS4 and the downstream antisense primer ITS5 generated approximately 600-700 bp DNA fragments. The PCR products were cloned by the TA cloning vector into the middle of two EcoRI sites of the pGEM T easy vector (Promega, USA) by following manufacturer's instructions to generate ITS1/5.8S/ITS2 region. To transform recombinant plasmids, the JM 109 strain of *Escherichia coli* was used as competent cells. Recombinant plasmids were screened by blue/white colour screening on X-gal containing plates and isolated from individual white colonies by a commercial plasmid purification kit (Thermo Fisher, USA). To check the success of the molecular cloning the DNA inserts were excised by digestion with EcoRI and electrophoresed on a 1.5% agarose gel.

Predicting the most stable secondary structure

All possible the most stable secondary structures of ITS1/5.8S/ITS2 regions for a total of 8 species sequences were modelled using the mfold structure prediction package of CLC RNA Workbench Version 6.2 (CLC bio, Denmark). Calculating free energies of all possible secondary structures and retaining one of the

lowest energies (i.e., the most stable) is the basis of predicting thermodynamically the most stable rRNA molecule (Chastain and Tinoco, 1991).

Phylogenetic analysis

The phylogenetic analysis was performed on the ITS1/5.8S/ITS2 sequence of each species. The aligned sequences were visually inspected for possible mutations and gaps. Cladistics analyses and the relationships were performed with a computer program (CLC Main Workbench) under both parsimony and maximum likelihood criteria using 1000 bootstrap replicates. The phylogenetic tree was constructed with the CLC Main Workbench program by the Neighbour-Joining method.

Results

A total of 8 species out of 11 samples from various ecological sites of Turkey were molecularly studied for the systematic utility of nuclear ribosomal DNA sequences by assessing phylogenetic relationships in genus *Cuscuta* (Cuscutaceae). Complete ITS nucleotide sequences (including ITS1, ITS2, and the 5.8S rRNA gene) and partial sequences of 18S and 26S ribosomal RNA gene were determined by molecular cloning.

The average length of the sequenced ITS region of studied *Cuscuta* species was approximately 665 bp, as seen in Table 1. In pairwise comparisons of aligned complete ITS1 and ITS2 nucleotide sequences, species of *Cuscuta* were similar in 92-99% of each other. The species names, EMBL accession numbers, DNA length of all 8 taxa used in this study are given in Table 1. Overall, 59 variable nucleotides were observed among the sequenced species. The highest divergence was observed on *C. hyalina* in a group with twenty-three indels (deletions or insertions). The alignment of the 8 taxa indicated that *C. babylonica* was the next divergence species with eighteen indels. Seventeen variable characters were observed between *C. babylonica* and *C. kotschyana* sequences as indels clustered at the same clade.

All possible predicted secondary structures of ITS regions were modelled for 8 species of *Cuscuta* (Figure 2). The utility of secondary structure's topology has been evaluated to resolve phylogenetic relationships at the species level. *C. monogyna* contained the most frequent hairpin structures on its ITS sequence having 13 hairpin secondary structures among the 8 species analysed. The remaining species have mostly consisted of a minimum 8 and maximum 11 hairpin structures (Figure 2). Our study clearly showed that the same species consistently form similar topology in secondary structure models (Figure 2 panel f and g).

To determine the degree of relationships and diversity between *Cuscuta* species phylogenetic analyses were performed. The resulting phylogenetic tree of ITS1 and ITS2 sequences gave a robust tree providing a strong resolution (Figure 3). The neighbour-joining tree was preferred since the tree offered more information and a better resolution with 1000 bootstrap values. The method divided 8 taxa into three major clusters. *C. kotschyana, C. babylonica, C. monogyna and C. hyalina* were clustered in the central largest group. *C. europaea* and *C. palaestina* were placed in the same clade because of the high level of sequence similarity.

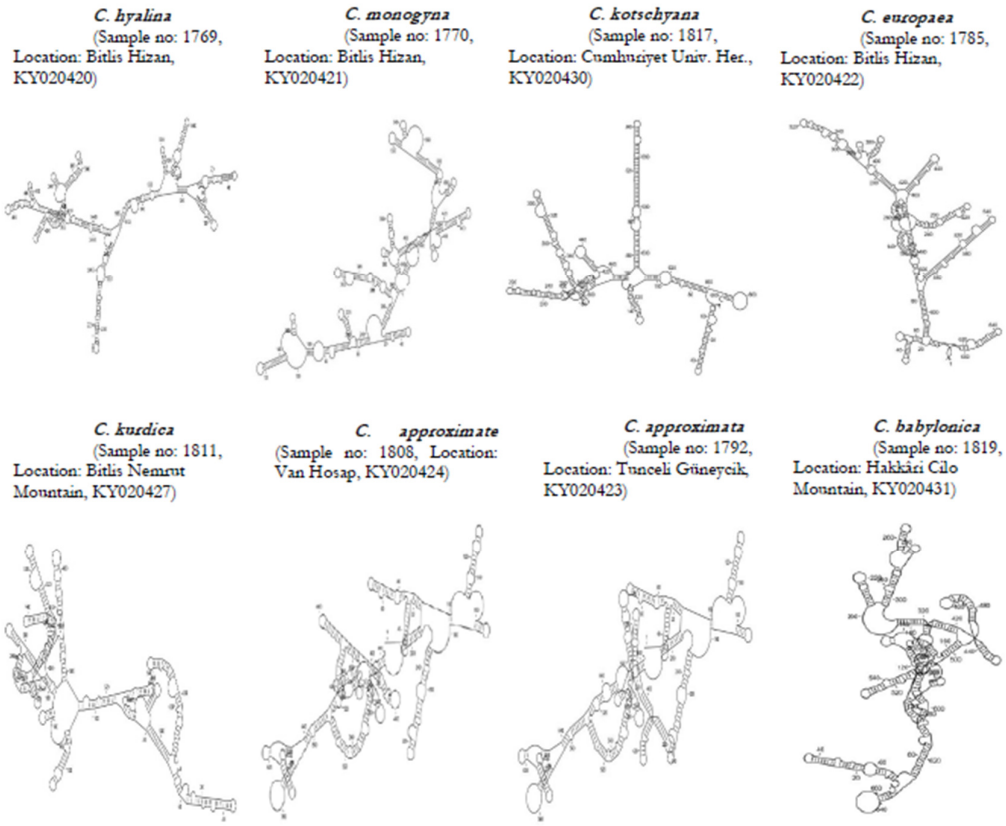


Figure 2. Secondary structures of ITS sequences of *Cuscuta* samples studied in this paper
Note them multitudinous hairpin structures of *C. monogyna*. of *C. hyalina* (a), *C. monogyna* (b), *C. kotschyana* (c), *C. europaea* (d), *C. kurdica* (e), *C. approximata* (f), *C. approximata* (g) *C. babylonica* (h).

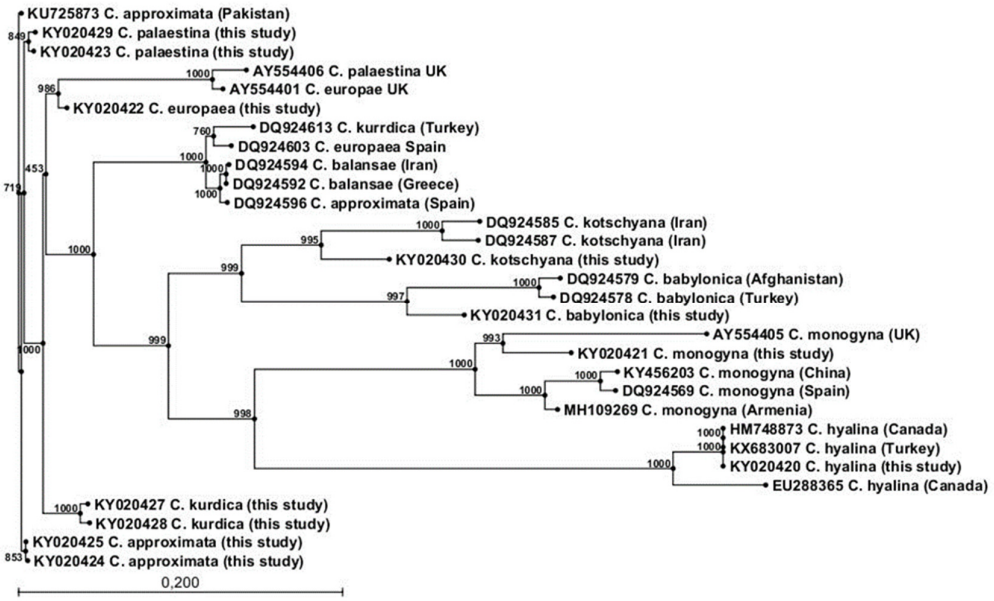


Figure 3. Phylogenetic analysis of the twelve ITS sequences observed among the species of *C. hyalina*, *C. monogyna. C. europaea, C. palaestina, C. approximata, C. kurdica, C. kotschyana* and *C. babylonica*
Numbers on the branches represent the 1000 bootstrap replications in which a given branch appears

Discussion

The molecular characteristics and the phylogenetic utility of ITS region of nuclear ribosomal DNA belonging to *Cuscuta* were analysed. ITS sequences were found suitable for phylogenetic comparisons at lower taxonomic levels. The present findings suggest that ITS sequences provide phylogenetically informative information and generate highly resolved trees with more strongly supported clades. To study phylogenetic relations among plant genera, the ITS sequences have been used widely in many weed and crop plants such as sorghum, maize, wheat, and cotton (Sonnate *et al.*, 2003).

The entire ITS region (ITS1-5.8S-ITS2) has been generated successfully using the reported ITS4 and ITS5 primers and templates of the total cellular DNA of *Cuscuta* spp. as shown in Figure 2. The ITS region of nrDNA contains 59 indels in *Cuscuta* with the length varying from 688 to 730bp. The traditional classification of *Cuscuta* species was consistent with the highly resolved phylogenetic tree constructed based on the ITS region. Based on ITS phylogeny, the 12 *Cuscuta* samples were differentiated into 8 main species.

The most stable secondary structure of ITS sequence of each defined species was constructed for all species studied in this research. Our study showed that the secondary structure of all studied species was exhibited diverse topology for each species which were enough to identify at the species level (Figure 2). It has been concluded that diverse species consistently form diverse topology in secondary structure models (Figure 2).

The combination of two molecular analyses (ITS sequencing and predicting the most stable secondary structure), was found successfully applicable in detecting and identifying dodders at the species level. Our results are in correlation with our previous study (Keskin *et al.*, 2017) conducted with the *Cuscuta* species collected from different sites of East Anatolia (Turkey). In recent years, many studies have been combining the knowledge of the prediction of the most stable secondary structures of the nucleic acids and the phylogenetic analyses of ITS structures to provide a more comprehensive understanding of the molecular basis of the species studied (Nicholas *et al.*, 2008).

Sequence analysis is very useful for phylogenetic analyses where morphological characters are inadequate (Yokoyama, 2000). Especially problems related to a diagnosis that cannot be resolved by taxonomic methods can be overcome by the evidence obtained from molecular data. One of the methods used for this purpose is ITS-PCR in the nrDNA region (Baldwin *et al.*, 1995). Recent developments in molecular biology allow the identification of plant species by identifying species-specific gene regions. ITS-PCR is the most reliable method for phylogenetic studies to be carried out at the genus and species level. For this reason, ribosomal DNA internal transcribed spacers (rDNA ITS) are frequently used in parallel with the functioning of the plant systematics and genomic regions they have in definition (Baldwin *et al.*, 1995).

Conclusions

Due to the reduced vegetative parts of parasitic plants, flower characters are generally used in the identification of *Cuscuta* species. Because sometimes these characters are also inadequate, for the diagnosis of *Cuscuta* L. genus, it is often necessary to combine molecular methods with morphological characters. Along with the evolutionary process, it is known that there are some variations between species and species in plants necessitating revision studies. In the present study, we determined that the secondary structure of the ITS sequences is highly conserved among the species in this study supporting successfully reconstructing the phylogenies at the species level. It has been concluded that the ITS sequences of nrDNA of *Cuscuta* provide enough data to identify and classify the economically relevant species along with the distinctive structural characters.

Authors' Contributions

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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