Identification of medicinal plants in the family Fabaceae using a potential DNA barcode ITS2

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Aim of the study: To test whether the ITS2 region is an effective marker for use in authenticating of the family Fabaceae which contains many important medicinal plants.

Materials and methods: The ITS2 regions of 114 samples in Fabaceae were amplified. Sequence assembly was assembled by CodonCode Aligner V3.0. In combination with sequences from public database, the sequences were aligned by Clustal W, and genetic distances were computed using MEGA V4.0. The intra- vs. inter-specific variations were assessed by six metrics, Wilcoxon two-sample tests and “barcoding gaps”. Species identification was accomplished using TaxonGAP V2.4, BLAST1 and the nearest distance method.

Results: ITS2 sequences had considerable variation at the genus and species level. The intra-specific divergence ranged from 0% to 14.4%, with an average of 1.7%, and the inter-specific divergence ranged from 0% to 63.0%, with an average of 8.6%. Twenty-four species found in the Chinese Pharmacopoeia, along with 39 species known for their use in traditional Chinese medicine, were successfully identified based on ITS2 sequences. In addition, ITS2 worked well, with over 80% of species and 100% of genera being correctly differentiated for the 1507 sequences derived from 1126 species belonging to 196 genera.

Conclusions: Our findings support the notion that ITS2 can be used as an efficient and powerful marker and a potential barcode to distinguish various species in Fabaceae.

1. Introduction

Fabaceae is the second largest family of medicinal plants, containing over 490 medicinal plant species, most of which have been used as traditional medicines. There are 31 species of medicinal plants belonging to 20 genera in the family Fabaceae that have been described in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2005), as well as numerous species that are included in the Japanese Pharmacopoeia. These species possess important medicinal properties and have been widely used as components of pharmaceutical products. For instance, Glycyrrhiza uralensis, Glycyrrhiza inflata, and Glycyrrhiza glabra, which are all generally used in traditional medicines, have inhibitory effects on HIV replication in vitro and anti-Fas antibody-induced hepatitis (Okamoto, 2000; Watanabe et al., 1996). Trigonella foenum-graecum has been shown to reduce blood glucose levels significantly (Vats et al., 2002). Additionally, plant materials from nearly 290 species belonging to 100 genera of Fabaceae have been reported to be toxic. For example, Acacia rigidula has been shown to contain appreciable levels of toxic alkaloids (Clement et al., 1998), and many of species of the genus Crotalaria contain pyrrolizidine alkaloids, which are toxic to mammals and birds (Williams and Molyneux, 1987).

Commonly used medicinal species and their adulterants are frequently found in the market together. For example, Astragalus membranaceus and Astragalus mongolicus are two of the most popular traditional Chinese medicines. However, Hedysarum polybotrys is often mistaken for Radix Astragali (Ma et al., 2002). Pueraria tuberosa is often used in traditional medicine in India, but there are at least three other botanical entities, Ipomoea mauritiana, Adenia hondala and Cynas circinata that are traded under the same name (Devaiah and Venkatasubramanian, 2008).

The identification of different species of Fabaceae is difficult when based solely on morphological characteristics (Hou et al., 2008; Newmaster and Ragupathy, 2009); additionally, some limitations in traditional taxonomy prevent this technique from meeting the complicated demands of species recognition (Maddison et al., 2007). As such, a method for the simple and accurate authentication of Fabaceae is indispensable.
Several DNA barcodes (matK, rbcL, psbA-trnH, ITS, rpoC1, etc.) have been developed for the identification of species (Kress and Erickson, 2007; Kress et al., 2009; Lahaye et al., 2008; Song et al., 2009; Newmaster and Ragupathy, 2009; Pang et al., 2010; Luo et al., 2010), but the barcodes for Fabaceae species are limited to a few genera and cannot be applied across the family (Edwards et al., 2008; Hollingsworth et al., 2009; Newmaster and Ragupathy, 2009). Recently, Plant Working Group has recommended rbcL and matK as core DNA barcodes (CBOL Plant Working Group, 2009). However, theoretically, nuclear DNA would provide more information for barcoding than organellar DNA (Chase and Fay, 2009). The effectiveness of ITS and ITS1 have been questioned in regard to improving the quality of primers to enhance their universality (Chase et al., 2007; Kress and Erickson, 2007; Chen et al., 2010). While, being part of ITS, ITS2 is relatively easy to be amplified using one pair of universal primers (Chiou et al., 2007; Chen et al., 2010). In addition, ITS2 has been found to provide taxonomic signatures in systematic evolution (Coleman, 2003, 2007; Schultz et al., 2005). The ITS2 region is also a promising potential molecular marker to be used for rapid taxonomic classification (Chiou et al., 2007; Chen et al., 2010).

In the current study, we utilize ITS2 as a DNA barcode to differentiate medicinal plants within the Fabaceae family in order to ensure their safe application in traditional uses.

### 2. Materials and methods

#### 2.1. Plant materials

In our study, 114 samples, which belonged to 85 species from 49 genera, were collected from large geographical areas in China between July 2007 and January 2008 (Online Resource 1). All plant species were identified by Professor Yulin Lin, Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Professor Quanru Liu, Beijing Normal University. The voucher samples were deposited in the herbarium of IMPLAD.

We have combined the sequences generated in our lab and those available from GenBank. The first dataset consisted of 184 important medicinal species and their adulterants as well as related species in Fabaceae (Online Resource 1, 2). These include 71 samples belonging to 29 species from 19 genera found in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2005). The second dataset consists of data from dataset 1 and all sequences from GenBank (Online Resource 1–3) belonging to Fabaceae samples. This dataset includes 1507 sequences from 1126 species representing 196 genera across all three subfamilies of Fabaceae and was used as a denser dataset to assess the identification efficiency of ITS2 sequences. Our datasets contain many closely related species (Table 1).

#### 2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted from silica gel-dried leaves or the medicinal materials bought from the markets including Trigonella foenum-graecum and Glycine max according to the protocol associated with the Plant Genomic DNA Kit (Tiangen Biotech Co., China). Polymerase chain reaction (PCR) amplification of the ITS2 region was carried out in a Peltier Thermal Cycler PTC200 (BioRad Lab Inc., USA) using approximately 30 ng of genomic DNA as a template in a 25-μl reaction mixture (1 × PCR buffer without MgCl₂, 2.0 mM MgCl₂, 0.2 mM of each dNTP, 0.1 μM of each primer (Synthesized by Sangon Co., China)), and 1.0 U of Taq DNA Polymerase (Biocolor BioScience & Technology Co., China). We designed universal primers and reaction conditions of the ITS2 region (Online Resource 4). The PCR products were run on a 1.0% agarose gel in 0.5 × TBE buffer and purified with the TIANgel Midi Purification Kit (Tiangen Biotech Co., China). The purified PCR products were sequenced on an ABI 3730XL sequencer (Applied Biosystems Inc.) using the amplification primers.

#### 2.3. Sequence alignment and data analysis

Contig assembly and the generation of consensus sequences were performed using CodonCode Aligner V 3.0 (CodonCode Co., USA). The ITS2 sequences from GenBank were subjected to Hidden Markov Model (HMM) model (Eddy, 1998, 2000) analysis to remove the conserved 5.8S and 26S (or equivalent) rRNA sequences (Keller et al., 2009). And we also used HMM based on well-curated fungal sequences to search for downloaded ITS2 sequences to remove the possible contaminated sequences of fungi. The sequences were then aligned using Clustal W (Thompson et al., 1994) and the genetic distances were computed using MEGA 4.0 according to the Kimura 2-Parameter (K2P) model (Tamura et al., 2007). The average intra-specific distance, coalescent depth and Theta were calculated to evaluate the intra-specific variation using the K2P model (Meyer and Paulay, 2005; Chen et al., 2010). The average inter-specific distance, the minimum inter-specific distance and Theta prime were used to represent inter-specific divergences (Meier et al., 2008; Meyer and Paulay, 2005; Chen et al., 2010). The distributions of intra- vs. inter-specific variability were compared using DNA barcoding gaps (Lahaye et al., 2008; Meyer and Paulay, 2005; Chen et al., 2010). Wilcoxon two-sample tests were performed as described previously (Kress and Erickson, 2007; Lahaye et al., 2008; Chen et al., 2010). The discriminatory power of ITS2 sequences for sister species was calculated by TaxonGAP V2.4 software (Naser et al., 2007; Slabbink et al., 2008). Two methods of species identification, including BLAST1 and the nearest distance method, were performed as described previously (Ross et al., 2008; Chen et al., 2010). In the BLAST1 method, correct identification means that the best BLAST hit of the query sequence is from the expected species; ambiguous identification means that the best BLAST hits for a query sequence were found to be those of several species including the expected species; incorrect identification means that the best BLAST hit of the query sequence is not from the expected species. In the distance method, correct identification means that the hit in our database based on the smallest genetic distances is from the same species as the that of the query; ambiguous identification means that several hits from our database were found to have the same smallest genetic distance to the query sequence; incorrect identification means that the hit based on the smallest genetic distance is not from the expected species.

### Table 1

The ITS2 sequences of plant materials used in the present study.

<table>
<thead>
<tr>
<th>ITS2 sequences</th>
<th>Dataset 1</th>
<th>Dataset 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sequences</td>
<td>184(92)*</td>
<td>1507(1126)</td>
</tr>
<tr>
<td>No. of sequences belonging to genera having more than one species</td>
<td>119(61)</td>
<td>1393(1028)</td>
</tr>
<tr>
<td>No. of sequences belonging to species having more than one samples</td>
<td>136(44)</td>
<td>605(224)</td>
</tr>
</tbody>
</table>

* The numbers of species to which these ITS2 sequences belong are shown in bracket.
DNA marker to use in identifying various species. Therefore, we first characterized the intra- and inter-specific variations in ITS2 sequences. The lengths of the ITS2 sequences used for the analyses ranged from 205 to 249 bps and 185 to 301 bps, in dataset 1 and dataset 2, respectively. In dataset 1, the inter-specific percentages of nucleotide differences ranged from 0% (Caragana rosea vs. Caragana sinica, Astragalus membranaceus vs. Astragalus mongholicus) to 42.3% (Phaseolus angularis vs. Phaseolus vulgaris), with an average of 13.4%. The percentage differences between individuals of the same species among the 184 Fabaceae sequences ranged from 0% (96 sequences) to 10.5% (Pueraria thomsonii), with an average of 1.2%. In dataset 2, the inter-specific percentages of nucleotide differences ranged from 0% (256 sequences) to 63.0% (Sophora alopecuroides var. alopecuroides vs. Sophora secundiflora), with an average of 6.2%. The percent differences between individuals of the same species among the 1507 Fabaceae sequences ranged from 0% (239 sequences) to 14.4% (Trifolium cherleri), with an average of 1.7%.

We then used six metrics to characterize inter- vs. intra-specific variations (Meier et al., 2008; Meyer and Paulay, 2005; Chen et al., 2010). As shown in Table 2, dataset 1 showed significant levels of inter-specific divergence within ITS2 sequences. Relatively lower levels of intra-specific divergence were found with calculations for all three metrics. An even larger difference was observed in dataset 2.

The inter-specific percentage differences among the Fabaceae species were greater than the intra-specific variations in dataset 1 and dataset 2. Therefore, the ITS2 region of the Fabaceae species, with lower levels of genetic divergence within species than between species, may be used as a genomic marker for the identification of these species.

3.2. Assessment of the intra- vs. inter-specific differences of ITS2 sequences

To perform a preliminary examination of inter- and intra-specific variation, we investigated the distribution of genetic distance in classes of 0.006 distance units. Only a slight overlap in inter/intra-specific variation of ITS2 was found in our study (Fig. 1). The inter-specific distance equaled to zero for only 1.2% and 0.5% of the samples in dataset 1 and dataset 2, respectively. Also, most of the Fabaceae species in our study were found to have a unique sequence in the ITS2. This will provide a useful way to authenticate different ITS2 species.

Wilcoxon two-sample tests also showed that the mean of the inter-specific divergences was significantly higher than that of intra-specific variations in both datasets (Table 3, P < 0.001). Therefore, ITS2 possessed intra- and inter-specific variation gaps.

3.3. Evaluation of the discriminatory power of ITS2 sequences for sister species

The software TaxonGap was used to allow the straightforward evaluation of the discriminatory power of individual genes in the identification scheme of dataset 1 (Fig. 2). For the sequences collected in this study, over 90.0% of the sequences had an inter-specific diversity that was larger than that of the intra-specific diversity, so relatively clear species boundaries were observed for ITS2 sequences. This data indicates that ITS2 could be used to identify the medicinal Fabaceae species described in the Chinese Pharmacopoeia. Yet, there were exceptions: 6.4% of the species (separability values = 0, see dark grey bar) had identical sequences with their sister-species for Astragalus membranaceus vs. Astragalus mongholicus, Caragana rosea vs. Caragana sinica and Pueraria lobata vs. Pueraria thomsonii (Fig. 2).

3.4. Testing the efficacy of ITS2 for authentication

ITS2 sequences generated in this study were used to build reference sequence libraries, and then the sequences obtained from samples with certain identity classifications were used to search the database. Finally, we were able to determine the species identities of the query sequences using BLAST1 and the nearest genetic distance method.

ITS2 performed well when using either BLAST1 or distance discrimination method. In dataset 1, ITS2 obtained 96.2% and 94.6% identification success rates at the species level for BLAST1 and distance discrimination method, respectively, with no ambiguous identification at the genus level for ITS2 alone. In dataset 2, the success rates of ITS2 exceeded 80.0% at the species level and reached up to 100% at the genus level, using the BLAST1 and the nearest genetic distance method (Table 4).

Table 5 shows that the variations among six large genera were discrepant and could only be analyzed individually. ITS2 worked well in Swartzia, in which 77 sequences representing 60 Swartzia species were tested, and ITS2 readily identified 97.4% of the sequences at the species level. In contrast, only 37.2% of sequences from Caragana that could be successfully identified by ITS2. As for the other four genera, the rate of successful identification with the

Table 2

Analysis of inter-specific divergence between congeneric species and intra-specific variations in ITS2 sequences in Fabaceae.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Dataset 1</th>
<th>Dataset 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>All inter-specific distance</td>
<td>0.1340 ± 0.1266</td>
<td>0.0860 ± 0.0568</td>
</tr>
<tr>
<td>Theta prime</td>
<td>0.1126 ± 0.1028</td>
<td>0.1029 ± 0.0894</td>
</tr>
<tr>
<td>The minimum inter-specific distance</td>
<td>0.0444 ± 0.0520</td>
<td>0.0526 ± 0.0783</td>
</tr>
<tr>
<td>All intra-specific distance</td>
<td>0.0117 ± 0.0237</td>
<td>0.0170 ± 0.0216</td>
</tr>
<tr>
<td>Theta</td>
<td>0.0105 ± 0.0217</td>
<td>0.0148 ± 0.0259</td>
</tr>
<tr>
<td>Coalescent depth</td>
<td>0.0154 ± 0.0308</td>
<td>0.0180 ± 0.0304</td>
</tr>
</tbody>
</table>

Table 3

Wilcoxon two-sample tests for distribution of intra- vs. inter-specific divergences.

<table>
<thead>
<tr>
<th>Data sources</th>
<th>No. of inter-specific distances</th>
<th>No. of intra-specific distances</th>
<th>Wilcoxon W</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dataset 1</td>
<td>171</td>
<td>86</td>
<td>15747.5</td>
<td>2.591 x 10^-30</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>35505</td>
<td>850</td>
<td>2343280.5</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 4
Comparison of authentication efficiency for ITS2 using different methods.

<table>
<thead>
<tr>
<th>Data sources</th>
<th>Methods of identification</th>
<th>No. of samples</th>
<th>Correct identification (%)</th>
<th>Incorrect identification (%)</th>
<th>Ambiguous identification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dataset 1</td>
<td>BLAST1</td>
<td>184</td>
<td>96.2(100)***</td>
<td>0(0)</td>
<td>3.8(0)</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>184</td>
<td>94.6(100)</td>
<td>0(0)</td>
<td>5.4(0)</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>BLAST1</td>
<td>1507</td>
<td>83.4(100)</td>
<td>0(0)</td>
<td>16.6(0)</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>1507</td>
<td>80.6(100)</td>
<td>0(0)</td>
<td>19.4(0)</td>
</tr>
</tbody>
</table>

*The percentage of samples correctly identified at the genus level for ITS2 sequences are shown in brackets.

Fig. 2. The heterogeneity and separability for individual taxa of ITS2 based on 184 representatives of the family Fabaceae by TaxonGap (Naser et al., 2007; Slabbinck et al., 2008).

ITS2 was around 60.0–80.0% at the species level, which is relatively powerful for taxonomic classification.

4. Discussion

A rapid and accurate method to authenticate species from the large family Fabaceae is very important to ensure the safe usage of drugs made from these medicinal herbs. To our knowledge, this is the first time that the ITS2 region was used to identify plant materials from Fabaceae with such a large sample size. ITS2 was found to be a sufficiently variable DNA region among Fabaceae species as determination by genetic divergences, and ITS2 also demonstrated a higher capability of successful discrimination. ITS2 can be a powerful marker for taxonomy studies, identifying species and solving taxonomic problems. Moreover, significant success had been achieved using ITS2 to differentiate species from many Fabaceae genera.

For example, satisfactory results were obtained when identifying Swartzia species; of the 60 Swartzia species used for this analysis, a successful identification was achieved for all but two species (Swartzia grandifolia and Swartzia longicarpa). A similar success rate was obtained in studies using Astragalus. In particular, the taxonomic status of Astragalus mongholicus and Astragalus membranaceus are not clear, and a consensus has not been reached on whether Astragalus mongholicus (Astragalus membranaceus var. mongholicus) is a variety of Astragalus membranaceus (Dong et al., 2003). The ITS2 sequence of Astragalus membranaceus is 100% identical to that of Astragalus mongholicus, so our results indicate that Astragalus mongholicus is a variety of Astragalus membranaceus, which is consistent with the results of Dong et al. (2003). Even in the genus Caragana, in which the species were poorly classified, ITS2 was still able to distinguish between some confusing species. Adaptation to arid and cold environments and the possible existence of hybrid species leads to considerable morphological diversity in Caragana species, resulting in the inability to define boundaries between the species using morphological characteristics. There are many controversies regarding the classical taxonomic classifications in Caragana, such as the species Caragana microphylla, Caragana davazamci and Caragana korshinskii (Hou et al., 2008). In the traditional taxonomists’ opinion, Caragana microphylla and Caragana davazamci are closely related genetically, while convergent evolution caused Caragana davazamci and Caragana korshinskii to have similar morphological characteristics; however, this classification has led to considerable taxonomic debate. The ITS2 sequence of Caragana davazamci is 100% identical to that of one sample of Caragana microphylla and is different from that of a Caragana korshinskii sample by only one base. Therefore, the molecular results support the view of the traditional taxonomists and are consistent with a previous study using sequences of trnL-F and ITS (Hou et al., 2006).

However, it should be noted that the taxonomic assignment of sequences from GenBank might not be accurate. For example, some morphologic characteristics of Caragana rosea and Caragana sinica are similar to each other; Caragana rosea has pinnate leaf that is almost the same as that of Caragana sinica, which makes the classification of these two species controversial. Furthermore, culti-
vars, wild varieties, and horticultural species also cause problems. *Arachis hypogaea* has exactly the same sequence as its tetraploid wild congeneric species, *Arachis monticola*. If we take these factors into account, the power of ITS2 in species discrimination might be estimated to be lower for some genera (for example, *Trifolium* and *Phaseolus*).

Still, ITS2 cannot solve all the species determination problems in Fabaceae. For example, in *Caragana, Caragana tibetica* and *Caragana ordosica* (Ma et al., 2003; Hou et al., 2006, 2008; Wojciechowski et al., 1993; Zhang, 2004) were found to have identical ITS2 sequences, but they were already reported to be two different species based on their ITS sequences (Hou et al., 2008). Thus, other DNA marker(s) might be valuable when investigating certain genus and broad plant taxa such that complete species identification could be achieved in Fabaceae.

Because there are multiple copies of ITS2 sequences in the plant genomes, it is questionable whether the sequence obtained through PCR would be stable and representative. However, we think the PCR-amplified copies could represent the dominant information of the repeated part of the ITS2 nuclear genome in individuals and that, in the vast majority of cases, ITS2 can be effectively treated as a single locus as Coleman described (2009).

In summary, we found that ITS2 was not only a standard phylogenetic marker, but it could also be useful for identity codes. Thus, we recommend ITS2 as a candidate barcode sequence in Fabaceae, or even in a broader range of plant taxa.

Using ITS2 as a DNA barcode would broaden our understanding of phylogenetics and population genetics in Fabaceae. We also propose that using ITS2 as a DNA barcode sequence to resolve problems in the classification of genera and species in Fabaceae is practical. We expect that applying the DNA barcoding technique to Fabaceae species identification will ensure a safe and stable supply of traditional medicines made from plant materials derived from Fabaceae. ITS2 might provide an entry point for quality control and assurance of medicinal plant materials used in research as well as industrial production, customs, and forensic examination.

5. Conclusion

In this study, ITS2 was examined for its usefulness in identifying medicinal species of Fabaceae. Our findings show that the ITS2 region can not only be used to identify 24 Fabaceae species in the Chinese Pharmacopoeia, along with another 66 species, including their adulterants, but can also correctly distinguish over 80.0% of species and 100% of genera from the 1507 sequences of Fabaceae species. Hence, ITS2 is a powerful and efficient tool for species identification of medicinal plants and even for a broad series of Fabaceae plant taxa, and ITS2 is a potential DNA barcode for Fabaceae.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2010.04.026.

References


Table 5

<table>
<thead>
<tr>
<th>Genera</th>
<th>Method</th>
<th>No. of sequences</th>
<th>No. of species</th>
<th>Success identification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifolium</td>
<td>BLAST1</td>
<td>267</td>
<td>226</td>
<td>62.6</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>267</td>
<td>226</td>
<td>59.6</td>
</tr>
<tr>
<td>Phaseolus</td>
<td>BLAST1</td>
<td>106</td>
<td>64</td>
<td>79.3</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>106</td>
<td>64</td>
<td>77.4</td>
</tr>
<tr>
<td>Caragana</td>
<td>BLAST1</td>
<td>43</td>
<td>28</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>43</td>
<td>28</td>
<td>37.2</td>
</tr>
<tr>
<td>Daviesia</td>
<td>BLAST1</td>
<td>46</td>
<td>44</td>
<td>82.6</td>
</tr>
<tr>
<td></td>
<td>Distance</td>
<td>46</td>
<td>44</td>
<td>78.3</td>
</tr>
<tr>
<td>Acacia</td>
<td>BLAST1</td>
<td>34</td>
<td>23</td>
<td>82.4</td>
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<tr>
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<td>67.6</td>
</tr>
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<td>BLAST1</td>
<td>77</td>
<td>60</td>
<td>97.4</td>
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<tr>
<td></td>
<td>Distance</td>
<td>77</td>
<td>60</td>
<td>97.4</td>
</tr>
</tbody>
</table>


