Evaluation of seven gene loci from the WRKY gene family in *Butia* (Becc.) Becc. species (Arecaceae) for future phylogenetic inference

Avaliação de sete loci gênicos da família de genes WRKY na *Butia* (Becc.) Becc. espécies (Arecaceae) para inferência filogenética futura

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**ABSTRACT**  
The WRKY genes belong to a family of transcriptional proteins that have been useful in the phylogenetic inference of plants, especially for representatives of the Arecaceae. This study aimed to expand the molecular data for species of the genus *Butia* (Arecaceae), through the WRKY2, WRKY6, WRKY7, WRKY12, WRKY16, WRKY19, and WRKY21 gene markers, using the species *Butia odorata* (Barb.Rodr.) Noblick, *Butia lallemantii* Deble & Marchiori, *Butia buenopolensis* Sant’Anna-Santos, and *Butia exilata* Deble & Marchiori. We obtained positive results in the amplifications for the gene regions of interest for WRKY6, WRKY7, and WRKY21 for the three individuals evaluated for the four species. For the WRKY2 gene, we only obtained amplifications from the individuals of the *B. buenopolensis*. The WRKY16 gene amplified for all species, except *B. buenopolensis*. On the other hand, the WRKY12 and WRKY19 gene
regions did not amplify for any of the individuals evaluated from the four species included in this study. With this work, we aimed to expand the molecular data of species belonging to the *Butia* genus, in order to infer the molecular phylogeny of species that occur naturally in Brazil in the future.

**Keywords:** Arecaceae, evolution, phylogenetic inference, WRKY transcription factor.

**RESUMO**
Os genes WRKY pertencem a uma família de proteínas transcricionais que tem se mostrado útil na inferência filogenética de plantas, em especial para representantes de Arecaceae. Este trabalho teve como objetivo ampliar os dados moleculares para as espécies do gênero *Butia* (Arecaceae), por meio dos marcadores gênicos WRKY2, WRKY6, WRKY7, WRKY12, WRKY16, WRKY19 e WRKY21, utilizando as espécies *Butia odorata* (Barb.Rodr.) Noblick, *Butia lallemantii* Deble & Marchiori, *Butia buenopolensis* Sant’Anna-Santos e *Butia exilata* Deble & Marchiori. Obteve-se resultados positivos nas amplificações para as regiões de interesse dos genes WRKY6, WRKY7 e WRKY21 para os três indivíduos das quatro espécies testadas. Para o gene WRKY2, obtivemos apenas amplificações dos indivíduos de *B. buenopolensis*. O gene WRKY16 amplificou para todas as espécies, com exceção de *B. buenopolensis*. Já as regiões gênicas WRKY12 e WRKY19 não amplificaram para nenhum dos indivíduos testados das quatro espécies incluídas neste estudo. Com este trabalho, objetivou-se ampliar os dados moleculares das espécies pertencentes ao gênero *Butia*, a fim de inferir futuramente a filogenia molecular das espécies que ocorrem naturalmente no Brasil.

**Palavras-chave:** Arecaceae, evolução, inferência filogenética, fatores de transcrição WRKY.

**1 INTRODUCTION**
The WRKY gene family is a family of proteins that act as transcription factors and are named after the most characteristic domain of these proteins, a region of 60 amino acids highly conserved among the genes that make them up, which allows them to bind to specific DNA sequences and regulate gene expression (Eulgem *et al.*, 2000). WRKY genes are widely and predominantly distributed in plants and are not found in animals (Zhang; Wang, 2005).

These genes are responsible for a variety of biological processes, including responses to biotic and abiotic stresses, embryogenesis, and defense
against pathogens (Dong *et al.*, 2003), as well as plant development through the regulation of starch and seed development (Garcia *et al.*, 2005), indicating that they play an important role in the evolution of this clade.

The WRKY sequences were used to develop 10 informative markers to estimate the genetic diversity of cultivar varieties of *Cocos nucifera* L (Mauro-Herrera *et al.*, 2006).

Understanding the extent of the presence of WRKY genes in the plant genome and their evolutionary importance, these genes began to be used in molecular phylogeny to infer evolutionary relationships among different plant species. Borrone *et al.* (2007), using representatives of the Malvaceae as an example of the usefulness of these genes in phylogenetic inference, reconstructed the intergeneric phylogenetic relationship of family representatives by comparing the sequence of WRKY genes in distinct species of the family, as well as the number and organization of WRKY genes in their genomes.

With evidence of the possible usefulness of these genes in phylogenetic inference, Merrow *et al.* (2009) used seven WRKY gene loci to infer the relationships within the Attaleinae subtribe (Arecaceae) and solve the long-standing puzzle of the evolution of *C. nucifera*, identifying the clade as a sister group of the genus *Syagrus* Mart. They used WRKY genes to support the evolutionary relationship of the clades, as well as the phylogenetic relationship of the other genera within the subtribe.

In this same study, the authors discuss the relationship between the American and African clades of genera within the Attaleinae subtribe, as well as point out the unquestionable monophyly of the subtribe, based on the phylogenetic analysis of these seven WRKY loci.

Subsequently, with the evident usefulness of WRKY loci in plant phylogenetic inference (Borrone *et al.*, 2007; Meerow *et al.*, 2009), Merrow *et al.* (2015) used six WRKY loci to discuss the evolution of taxa in the Cocoseae tribe (Arecaceae), as well as the biogeographic history of the tribe through parsimony, maximum likelihood, and Bayesian analysis. Using this methodology, the authors
demonstrated the monophyly of the tribe and identified the Attaleinae subtribe as the oldest among the three belonging to the mentioned tribe.

Merrow et al. (2015) also confirms that the genera Butia (Becc.) Becc and Jubaea Kunth are sister groups, supporting the relationship already pointed out in other phylogenetic studies with the genera (Baker et al., 2009; Meerow et al., 2009), and point to the emergence and evolution of the clades in the region between the Andes Mountains Range (Chile) and Brazil.

The genus Butia is native from South America (Noblick, 2010) and belongs to the subtribe Attaleinae of the Arecaceae family (Dransfield et al., 2008). Currently, the genus Butia comprises twenty-two species with an accepted taxonomic position (Azambuja; Pereira, 2022).

Azambuja and Pereira (2022) point out that, although the genus Butia and its species have been known to science for a long time, the relationships among them are still poorly explored, and they suggest expanding research to understand the evolutionary history of the genus and its species.

Thus, the use of WRKY genes through the comparison of sequences of the seven gene loci evaluated in this study may provide information on the evolutionary relationship between species. Given this information, the aim of this study was to evaluate the amplification of seven WRKY gene markers for the two species mentioned by Meerow et al. (2009), Butia odorata (Barb.Rodr.) Noblick and Butia lallemantii Deble & Marchiori, with individuals from populations in their natural occurrence areas in Rio Grande do Sul State, and to expand the molecular data for two more species of the genus Butia that do not yet have molecular data for these genes: Butia buenopolensis Sant’Anna-Santos and Butia exilata Deble & Marchiori.

2 MATERIALS AND METHODS

2.1 SPECIES AND GENETIC MARKERS USED

One of the target species of this study is endemic to Brazil, with B. buenopolensis found in the state of Minas Gerais (Sant’Anna-Santos, 2021). The other species are cited as endemic to Rio Grande do Sul State and Uruguay
(Soares et al., 2014) and were collected in their natural occurrence places, with *B. lallemantii* collected in the municipality of Alegrete/RS, *B. odorata* collected in the municipality Manoel Viana/RS, and *B. exilata*, is also endemic to Brazil, were collected in the municipality Sarandi/RS, in order to sample the populations of natural occurrence in Rio Grande do Sul (Figure 1).

Figure 1 – Map of the sampled locations for *B. odorata*, *B. lallemantii*, and *B. exilata* species.

For this study, primers for seven gene loci belonging to the WRKY gene family were evaluated, following the methodology of Meerow et al. (2009). These authors expanded the use of these primers to the subtribe Attaleinae (Areaceae) based on primers designed by Mauro-Herrera et al. (2007). The sequences of the markers used in this study are described in Table 1.
Table 1 – Primer sequences (WRKY primer sequences (5' to 3')) used for amplification of four species of the genus *Butia* (Becc.) Becc.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRKY2</td>
<td>ACAATCACCCACGGCTTCTCA</td>
<td>ATTCACACACCCGGATGCTTC</td>
</tr>
<tr>
<td>WRKY6</td>
<td>F1: CCAAACCAAGGGATGTTTCAGC</td>
<td>R1: CCTACACGGGCAACCCAGCATT</td>
</tr>
<tr>
<td>WRKY7</td>
<td>F2: ACCCAAGCTCCACACA</td>
<td>R2: TCACGGGCTTGGATCAT</td>
</tr>
<tr>
<td>WRKY12</td>
<td>F2: GGTTGCTCAACACTCT</td>
<td>R3: TGCCCTCTCCACATGCTTTC</td>
</tr>
<tr>
<td>WRKY16</td>
<td>F1: AGCCGTCAAAAACAGCCCATT</td>
<td>R1: CAAGCACGGCAAGGTATCA</td>
</tr>
<tr>
<td>WRKY19</td>
<td>F3: AACACCACAAATCCACAGGAT</td>
<td>R3: TCCACTCTGTCTTCAACAGAACAAC</td>
</tr>
<tr>
<td>WRKY21</td>
<td>F1: AGAGTAACCCTGCCCCAGCA</td>
<td>R1: GCCACCTCTGACCTGAAA</td>
</tr>
</tbody>
</table>

Source: Meerow et al. (2009).

The biological material used to obtain nuclear DNA of *B. buenopolensis* was accessed through herbarium specimens deposited in Brazilian institutions (MBM - Herbarium of the Municipal Botanical Museum of Curitiba - Holotype: MBM 323217; Isotypes: Herbarium of the Ecological Reserve of IBGE - IBGE 83300; DIAM Herbarium- Federal University of Vale do Jequitinhonha e Mucuri-DIAM 8256; UFG Herbarium - Federal University of Goiás - UFG 70074), which provided leaf samples of the specimens for research without harming the integrity of the herbarium specimens.

The DNA of *B. exilata* was obtained from leaf samples collected at the natural occurrence site of the species (Sarandi, northwest of Rio Grande do Sul State/BR) in 2019, which were stored at -20°C until the moment of extraction.

For the other species (*B. odorata* and *B. lallemantii*), biological material was collected at the natural occurrence sites of each one (Manoel Viana and Alegrete, respectively), allowing for DNA extraction from fresh leaf tissue. Besides, DNA extraction was performed on three different individuals from the same collected population, except for *B. buenopolensis*, for which DNA was extracted from the four specimens deposited in the herbarium.
2.2 GENOMIC DNA EXTRACTION AND QUANTIFICATION

For the genomic DNA extraction of *B. buenopolensis*, the commercial Qiagen Plant MiniKit® (Hilden, Germany) was used following the manufacturer's standard protocol. For the extraction of DNA from the other species, the commercial TRANS® plant extraction kit (Transgen Biotech) was also used following the manufacturer's protocol. For both kits, approximately 100 mg of pinnae, excluding the mibrid, were used for both fresh and herbarium samples. The NanoVue TMPlus spectrophotometer was used for DNA quantification, and the integrity of the samples was verified by 1% agarose gel electrophoresis, stained with GelRedTM (BiotiumVR), and visualized under UV light.

2.3 POLYMERASE CHAIN REACTION (PCR)

The amplification of the target genes in this study was performed by Polymerase Chain Reaction (PCR), using specific primers for each region of interest in the nuclear genome of the target species (described in table 1).

To amplify the desired fragments, the PCR reactions were adapted according to Meerow *et al.* (2009), with a final volume of 25 µL per reaction. In each reaction, 15 µL of HotStarTaq® Master Mix- Qiagen (2.5 units of Taq DNA Polymerase; 1.5 mM MgCl₂; 200 µM of each dNTP), 1 µL of MgCl₂, 1 µL of the forward primer (concentration of 10 µM), 1 µL of the reverse primer (concentration of 10 µM), 1 µL of template DNA (in a concentration of 25-35 ng/μL of diluted DNA extraction, except for *B. buenopolensis*, which was not diluted due to the low amount of DNA per ng/μL, and 6 μL of ultrapure water (DNAse free) were added to complete the total volume of 25 µL per reaction.

The amplifications were performed using the BIO-RAD T100 TouchTM thermocycler, following the following cycle: an initial step at 95°C for 15 minutes for DNA denaturation and inactivation of the Taq polymerase inhibitor; subsequently, 39 cycles of 95°C for 30 seconds for denaturation, 1 minute at 61.1°C for primer annealing and 1 minute of extension at 72°C were executed. After completing the 39 cycles, a final extension step at 72°C for 2 minutes was performed.
For confirmation of amplification, PCR products were evaluated on 1% agarose gel prepared with 1X TBE buffer, stained with GelRedTM (BiotiumVR) and visualized under UV light. To estimate the size of the amplified fragments, a 100 base pair molecular weight marker (Ludwig Biotec) was used.

After confirming the amplification of the target loci, the PCR products were purified using the commercial PCR purification kit Trans® Transgen Biotech following the manufacturer’s protocol. The samples were then sent for sequencing service at the ACTGene laboratory (Ludwig Biotec) for further analysis and use in the phylogenetic inference of the Butia species targeted by this research.

3 RESULTS AND DISCUSSION

The amplification tests of the markers were performed on four species belonging to the Butia genus (Arecaceae): B. odorata, B. lallemantii, B. buenopolensis, and B. exilata. Two of these species already have sequences deposited in the GENBANK database (NCBI, 2023) for the listed genes: B. odorata and B. lallemantii. Two species that do not have molecular data cited in the literature were also included: B. buenopolensis and B. exilata.

After genomic DNA extraction from leaf tissue, the material was diluted in a final volume of 50 μL, and the DNA quantification results obtained for all analyzed individuals are shown in Table 2.

When comparing the results obtained among the four analyzed species, lower DNA concentration values were observed for individuals of B. buenopolensis, whose DNA was extracted from herbarium material, when compared to the amount of DNA obtained from fresh or frozen leaf tissue samples from the other species.
Table 2 – DNA quantification of each individual in ng/μL of the final concentration of 50μL of obtained DNA.

<table>
<thead>
<tr>
<th>Species (individual identification)</th>
<th>DNA Quantity (ng/μL)</th>
</tr>
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<tbody>
<tr>
<td>B. buenopolensis (BB-DIAM)</td>
<td>13,9 ng/μL</td>
</tr>
<tr>
<td>B. buenopolensis (BB-UFG)</td>
<td>32 ng/μL</td>
</tr>
<tr>
<td>B. buenopolensis (BB-MBM)</td>
<td>11,5 ng/μL</td>
</tr>
<tr>
<td>B. buenopolensis (BB-IBGE)</td>
<td>43,5 ng/μL</td>
</tr>
<tr>
<td>B. exilata (BEX-1)</td>
<td>81,5 ng/μL</td>
</tr>
<tr>
<td>B. exilata (BEX-40)</td>
<td>99,5 ng/μL</td>
</tr>
<tr>
<td>B. exilata (BEX-47)</td>
<td>132 ng/μL</td>
</tr>
<tr>
<td>B. lallemantii (BL-2)</td>
<td>59,5 ng/μL</td>
</tr>
<tr>
<td>B. lallemantii (BL-3)</td>
<td>104,5 ng/μL</td>
</tr>
<tr>
<td>B. lallemantii (BL-4)</td>
<td>73 ng/μL</td>
</tr>
<tr>
<td>B. odorata (BO-1)</td>
<td>93,5 ng/μL</td>
</tr>
<tr>
<td>B. odorata (BO-2)</td>
<td>73,5 ng/μL</td>
</tr>
<tr>
<td>B. odorata (BO-3)</td>
<td>82,5 ng/μL</td>
</tr>
</tbody>
</table>

Source: Authors.

In the evaluation of the integrity of the bands formed in 1% agarose gel from the extracted DNA, it was noticed that the DNA extracted from fresh leaf material of the species B. lallemantii and B. odorata (Figure 2-a) formed more defined bands in comparison to the bands formed in the species B. buenopolensis and B. exilata (Figure 2-b), indicating that DNA obtained from fresh biological material is more preserved compared to dehydrated and/or frozen material.
Figure 2 – 1% agarose gel for the evaluation of the integrity of the template DNA obtained from the species: a. *B. lallemantii* and *B. odorata* species; b. *B. buenopolensis* and *B. exilata* species.

When compared the values of DNA quantification and integrity on agarose gel between *B. buenopolensis* and *B. exilata*, although degraded, the DNA of *B. exilata* showed higher numbers in quantification and more defined bands compared to *B. buenopolensis*, indicating that frozen samples of *Butia* species may keep the DNA more preserved than dehydrated samples. However, DNA obtained from both species provided satisfactory results, as shown below.

The WRKY genes have been shown to be highly efficient in the phylogenetic reconstruction of plants, mainly due to the high conservation of these genes in the genome. In Arecaceae Meerow *et al.* (2009) pointed out that using these seven WRKY genes in the phylogenetic inference of the Attaleinae subtribe, *Syagrus* genus is a sister group of *C. nucifera*, answering an old question about the evolution of *C. nucifera*.

However, our results differ from those obtained in Meerow *et al.* (2009) study, where the authors amplified all seven gene regions used for all species of *Butia*. The Table 3 shows the relationship among the species included in our research and the tested WRKY loci, with results related to amplified and non-amplified loci through PCR.
Table 3 – Relationship between species and target gene loci: X: amplified; -: not amplified.

<table>
<thead>
<tr>
<th>Species</th>
<th>WRKY 2</th>
<th>WRKY 6</th>
<th>WRKY 7</th>
<th>WRKY 12</th>
<th>WRKY 16</th>
<th>WRKY 19</th>
<th>WRKY 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. buenopolensis</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>B. exilata</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>B. lallemantii</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>B. odorata</td>
<td>-</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
</tbody>
</table>

Source: Authors.

Regarding the four species sampled in this study, we obtained amplifications in all three individuals of *B. buenopolensis* for the genes WRKY2, WRKY7, and WRKY21. For the gene WRKY6, only two individuals amplified this gene region (Figure 3). One of the four individuals of *B. buenopolensis* (individual: BB-MBM), which presented a lower quantity of DNA (in ng/μL), did not amplify any of the tested genes, possibly influenced by the low quantity and quality of DNA. Thus, only the remaining three individuals were used.

Figure 3 – Image of 1% agarose gel for the evaluation of amplification of the genes of interest for the species *B. buenopolensis*.

Source: Authors.

Regarding *B. lallemantii* and *B. odorata*, we succeeded in amplifying genes WRKY6 and WRKY7 in all three tested individuals of each species, and for *B. exilata*, only two individuals amplified these genes (Figure 4).
For the genes WRKY6 and WRKY7, we obtained amplifications for all three individuals of *B. lallemantii*, *B. odorata*, and *B. exilata* species (Figure 4). For *B. buenopolensis*, we did not obtain any amplification for the gene WRKY16 in any of the tested individuals.

For the genes WRKY16 and WRKY21, we obtained amplifications for all three individuals of *B. lallemantii*, *B. odorata*, and *B. exilata* species (Figure 5). For *B. buenopolensis*, we did not obtain any amplification for the gene WRKY16 in any of the tested individuals.

Although there are sequences of the species *B. lallemantii* and *B. odorata* deposited in the GENBANK database (NCBI, 2023), originating from the WRKY genes sequencing performed by Meerow et al. (2009), for the species *B. lallemantii* and *B. odorata*, in our study, we were not successful in amplifying the...
WRKY2, WRKY12, and WRKY19 gene loci, for both species sampled by Meerow et al. (2009) and to two new species included.

In Meerow et al. (2015), the authors used six of the seven WRKY genes used in Meerow et al. (2009), indicating that they removed the gene WRKY2 due to the inability to amplify this region in some Bactris Jacq. ex Scop species. In addition, they point out that the primer designed for the WRKY2 gene region amplified the WRKY12 gene region in the same genus. Thus, the authors suggest a possible recent paralogy of these two genes.

Considering that the primers used by Meerow et al. (2009; 2015) were developed from sequences isolated from C. nucifera (Mauro-Herrera et al. 2006) and applied to amplification tests in other genera and species of the tribe Cocoseae and subtribe Attaleinae, it indicates that perhaps the closest clades from C. nucifera have more success in amplifying these gene regions when compared to evolutionarily more distinct or more recent clades in the evolutionary lineage of the family.

An interesting factor about the WRKY2 and WRKY12 genes is that using the same DNA extraction and PCR methodology, Oliveira-Neves et al. (2022) successfully amplified these two gene regions for the hybrid palm xButyagrus nabonnandii (Prosch.) Vorster, using DNA extracted from both leaf and stem from three different individuals.

However, it should be noted that xB. nabonnandii has Syagrus romanzoffiana Mart. as one of its parental species (a genus that was identified as sister group of C. nucifera based on the same seven WRKY genes) and B. odorata, from which we did not obtain any positive amplification results for these two loci.

The presence of homologous WRKY genes in species from different clades may indicate that these genes arose before the divergence of the clades, while the absence of homologous genes in some clades may indicate that these genes arose after the divergence of the clades.

However, it should be noted that the non-amplification of the genes of interest in this research may have occurred due to the adopted methodology. The
future use of other methodologies may alter the results obtained with the target species.

Thus, amplification and sequencing, and subsequently nucleotide analysis of WRKY genes can provide information on gene duplication and loss events that occurred during evolution.

The same hypothesis can be applied to the WRKY2 and WRKY19 loci, but this can only be confirmed by expanding the use of WRKY genes to other species belonging to the *Butia* genus.

Given the results obtained regarding the non-amplification of these three genes in the four species included in the present study, we assessed a different annealing temperature gradient for these three genes compared to the other loci.

For the annealing temperature gradient of the primers of these three loci (WRKY2, WRKY12, and WRKY19) during PCR, we assessed three different temperatures: 61.1°C, 58.8°C, and 56.9°C. However, we were not successful in amplifying them. Nevertheless, it is worth noting that Oliveira-Neves *et al.* (2022) reported successful amplification of all three individuals of *xButyagrus nabonrandii* at a primer annealing temperature of 61.1°C, which is the same temperature used in our study. This result reinforces the hypothesis that new strategies will need to be employed to attempt to amplify these genes.

4 CONCLUSION

Regarding the use of herbarium samples in this research, which was the only resource available to access the genetic material of *B. buenopolensis*, it was a valid resource that provided satisfactory results as shown above.

Based on the above, it is also important to highlight the role of herbaria as a source of biological resources for our biodiversity.

With the resolutions outlined in this work, our objective was to expand the molecular data of species belonging to *Butia* that were not sampled in Meerow *et al.* (2009), using the same gene loci, in order to infer in the future the molecular phylogeny of species that occur naturally in Brazil, especially the group of native species in the Southern region from Brazil (with natural occurrence in Paraná,
Santa Catarina, and Rio Grande do Sul States). Brazil has the highest number of *Butia* species, and it is considered the center of diversity of this genus.

However, the results obtained so far lead us to consider the exclusion of the WRKY2, WRKY12, and WRKY19 genes in future phylogenetic inferences of the genus *Butia*.

Other species belonging to the genus will be included in this research, namely *B. catarinensis* Noblick & Lorenzi; *B. microspadix* Burret; *B. pubispatha* Noblick & Lorenzi; *B. witeckii* K. Soares & S. Longhi. After evaluating the new results, we will be able to discuss with greater certainty the exclusion of these three loci if the results remain non-amplified.

**ACKNOWLEDGEMENTS**

This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. We would like to thank the Federal University of Pampa (UNIPAMPA) and the curators of the herbaria mentioned above who provided the material for this research to be conducted.
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