

Article

Revisiting the Genetic, Taxonomic and Evolutionary Aspects of Chagas Disease Vectors of the *Triatoma phyllosoma* Subcomplex (Hemiptera, Triatominae)

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Abstract: *Triatoma bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* are species that have great epidemiological importance in the transmission of Chagas disease in Mexico. However, there is no consensus regarding the specific status of these species, since they appear in various articles as species, subspecies and even subgenera. Thus, we revisited genetic, taxonomic and evolutionary data that allowed us to assess and discuss the specific status of these six species of the *T. phyllosoma* subcomplex. Phylogenetic studies were performed with nuclear (*18S*, *28S*, *ITS-2*) and mitochondrial (*16S*, *cytb*, *COI*, *COII*, *12S*) markers deposited in GenBank. In addition, data from experimental crosses were pooled and the genetic distance to the *cytb* gene was calculated. The phylogenetic reconstruction enabled us to rescue the six species as independent lineages. Post-zygotic reproductive isolation barriers (sterility and/or hybrid collapse) were observed for some experimental crosses. Although the other experimental crosses did not allow us to characterize reproductive barriers, these species showed high genetic distances in relation to the *cytb* gene (ranging from 4.6% to 14.9%). Thus, based on the revisited literature data, we confirmed the specific status of these six species of the *T. phyllosoma* subcomplex based on the phylogenetic and biological concepts of the species.

Keywords: kissing bugs; triatomines; phylogenetic systematics; experimental crosses



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1. Introduction

The Chagas disease vectors of the Triatomini tribe (Hemiptera, Triatominae) have been grouped into eight complexes and nine subcomplexes (Figure 1) [1–5]. Although these groupings are not recognized by the International Code of Zoological Nomenclature [6], it has been suggested that they should represent natural groups (monophyletic) [7]. The *Triatoma phyllosoma* subcomplex is composed of the species *T. bassolsae* Alejandro Aguilar et al., 1999; *T. bolivari* Carcavallo, Martínez and Pelaez, 1987; *T. longipennis* (Usinger, 1939); *T. mazzottii* (Usinger, 1941); *T. mexicana* (Herrich-Schaeffer, 1848); *T. pallidipennis* (Stål, 1872); *T. phyllosoma* (Burmeister, 1835); *T. picturata* (Usinger, 1939) and *T. ryckmani* Zeledón and Ponce, 1972 [1]. With the exception of *T. ryckmani*, all the species of the *T. phyllosoma* subcomplex are endemic to Mexico [8].

Triatoma bassolsae, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* are species that have great epidemiological importance in the transmission of Chagas disease in Mexico, representing more than 60% of vectorial transmissions of *Trypanosoma*

cruzi (Chagas, 1909) (Kinetoplastida, Trypanosomatidae) to humans [9]. In addition to their epidemiological importance, these insects have a complex taxonomy, since they were once considered a single species with genetic and morphological polymorphism and/or subspecies of *T. phyllosoma* [10].

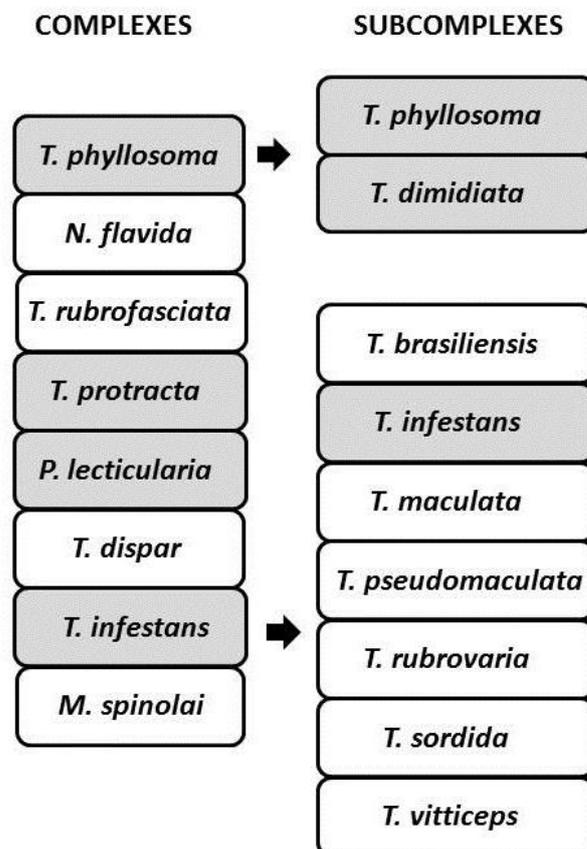


Figure 1. Complexes and subcomplexes that are used to group the species of the Triatomini tribe. The shaded groupings represent species that have already been reported in Mexico.

Triatoma bassolsae was described in 1999 as a species of the genus *Triatoma* Laporte, 1832 [11]. In 2000, Carcavallo et al. [12] suggested the reclassification of the species to the genus *Meccus* Stål, 1859, and recently Justi et al. [7] and Cesaretto et al. [13] demonstrated that it is a species of *Triatoma*. *Triatoma longipennis* was described 1939 as a species of the genus *Triatoma* [14]. In 1944, it was considered as a subspecies: *T. p. longipennis* [15]. In 2000, Carcavallo et al. [12] suggested that the subspecies was a species and grouped it into the genus *Meccus*. Recently it was demonstrated that this species belongs to the genus *Triatoma* [7,13]. *Triatoma mazzottii* was described 1941 as a species of the genus *Triatoma* [16]. In 1943/1944, it was considered a subspecies (*T. p. mazzottii*) [15,17]. Later, it came to be considered as a species and was classified in the genus *Meccus* [12] and recently it has been regrouped into the genus *Triatoma* [7,13].

Triatoma pallidipennis was described in 1872 as a species of the genus *Triatoma* [18]. In 1943/1944, it was considered a subspecies (*T. p. pallidipennis*) [15,17]. In 2000, it started to be considered a species and was placed in the genus *Meccus* [12]. Recently Justi et al. [7] and Cesaretto et al. [13] demonstrated that it is a species of *Triatoma*. *Triatoma phyllosoma* was described in 1835 as a species of the genus *Conorhinus* [19]. In 1930, it came to be considered a species of the genus *Triatoma* [20]. In 2000, it was classified in the genus *Meccus* [12] and recently it has been regrouped into the genus *Triatoma* [7,13]. Finally, *T. picturata* was described in 1939 as a species of the genus *Triatoma* [14]. In 1943/1944, it was considered a subspecies (*T. p. picturata*) [15,17]. In 2000, Carcavallo et al. [12] suggested

that the subspecies was a species and grouped it into the genus *Meccus*. Recently it was demonstrated that this species belongs to the genus *Triatoma* [7,13].

As demonstrated above, the generic status of the *T. phyllosoma* subcomplex species has also been widely discussed. The first species of this genus was described as *Conorhinus phyllosoma* Burmeister, 1835 [19]; in 1859 the species was transferred to the genus *Meccus* [20]; in 1930 it was transferred to the genus *Triatoma* [21]; in 2000 the genus *Meccus* was revalidated based on morphological data [12] (alteration corroborated by Hypsa et al. [22] through molecular studies); and in 2014 the genus *Meccus* was synonymized with *Triatoma* using more sophisticated phylogenetic reconstruction methods [7] (generic alteration recently confirmed by experimental crosses [13]).

Recently, Rengifo-Correa et al. [23] proposed an identification key for the *T. phyllosoma* species group (involving species of the *T. phyllosoma* and *T. dimidiata* subcomplexes) and suggested that *T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* should be considered species. However, there is no consensus among researchers between the specific status of these species, since they appear in articles as species [23], subspecies [24,25] and even subgenera [26,27]. Thus, we revisited genetic, taxonomic and evolutionary data that allowed us to assess and discuss the specific status of these six species of the *T. phyllosoma* subcomplex.

2. Materials and Methods

Sequences of eight molecular markers obtained in GenBank (mitochondrial markers: *16S*, *cytb*, *COI*, *COII* and *12S*; nuclear markers: *18S*, *28S* and *ITS-2*) (Table 1) were used for the *T. phyllosoma* subcomplex species (*T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata*) and for two *Triatoma* species (*T. brasiliensis* Neiva, 1911 and *T. vitticeps* (Stål, 1859)), which were designated as an outgroup (Table 1). The sequences were submitted to the MEGA X program [28] and aligned using the muscle method [29]. The alignments of each marker were concatenated by name using the Seaview4 program [30] and converted with the Mesquite program [31], resulting in an alignment with eight taxa and 5556 nucleotides.

Table 1. GenBank access codes of sequences used in the molecular analysis of the *T. phyllosoma* subcomplex species and respective nucleotide substitution models. - represents genes that have not been sequenced.

Species	Molecular Markers (Substitution Models)							
	<i>16S</i> (GTR + I + G)	<i>18S</i> (HKY +I)	<i>28S</i> (HKY)	<i>Cytb</i> (HKY + G)	<i>COI</i> (GTR + I)	<i>COII</i> (HKY)	<i>ITS-2</i> (HKY)	<i>12S</i> (GTR)
<i>T. phyllosoma</i> subcomplex								
<i>T. pallidipennis</i>	KC249045	AJ243330	-	DQ198814	-	-	AJ286882	AF394522
<i>T. longipennis</i>	KC249031	-	KC249177	KC249267	KC249357	KC249452	KC698909	-
<i>T. mazzottii</i>	AY035446	AJ243333	-	DQ198816	DQ198805	-	KC698911	-
<i>T. picturata</i>	AY035447	AJ243332	-	DQ198817	-	-	KC698910	-
<i>T. phyllosoma</i>	-	AJ243329	-	DQ198818	DQ198806	-	KC698912	-
<i>T. bassolsae</i>	-	-	-	MK317878	-	-	MK248256	-
Outgroup								
<i>T. brasiliensis</i>	KC248985	AJ421957	KC249145	KC249239	KC249318	KC249413	-	AF021187
<i>T. vitticeps</i>	KC249087	KC249132	KC249220	KC249303	KC249396	KC249491	-	AF021217

The concatenated alignment was partitioned for each marker and the best nucleotide substitution model (lowest Akaike information criterion value) was individually determined in the jModelTest 2 program [32] (Table 1). Data were submitted to MrBayes 3.2 [33] for phylogenetic reconstruction using a Bayesian approach, with a total of 100 million generations. Trees were sampled every 1000 generations in two independent runs, with

burn-in adjusted to 25%. The Tracer v. 1.7 program [34] was used to verify the stabilization (ESS values above 200) of the sampled trees and the generated phylogenetic tree was viewed and edited in the FigTree v.1.4.4 program [35], being rooted at the midpoint. A concatenated sequence tree was produced based on the mitochondrial and nuclear genes once the concatenation approach had yielded more accurate trees, even when the concatenated sequences had evolved with very different substitution patterns [36]. The genetic distance matrix between the *T. phyllosoma* subcomplex species was obtained in the MEGA X program 21 based on the *cytb* sequences (Table 2) using the Kimura 2-parameter distance model [37]. The use of only one specimen of each species in the matrix was justified because the objective of this genetic distance analysis was to assess the taxonomic status of each of the six taxa of this subcomplex (interspecific) and not to carry out population studies (intraspecific).

Table 2. Genetic distance matrix for the cytochrome b gene.

Species	1	2	3	4	5	6	7	8
1 <i>T. pallidipennis</i>								
2 <i>T. longipennis</i>	0.104							
3 <i>T. mazzottii</i>	0.136	0.102						
4 <i>T. picturata</i>	0.090	0.106	0.148					
5 <i>T. phyllosoma</i>	0.124	0.091	0.122	0.147				
6 <i>T. bassolsae</i>	0.046	0.099	0.149	0.084	0.132			
7 <i>T. brasiliensis</i>	0.315	0.296	0.326	0.360	0.276	0.336		
8 <i>T. vitticeps</i>	0.295	0.267	0.295	0.265	0.267	0.256	0.302	

3. Results and Discussion

The phylogenetic reconstruction, obtained by combining different mitochondrial and nuclear markers, could be used to rescue the six species of the *T. phyllosoma* subcomplex as independent lineages with strong bootstrap values (values $\geq 70\%$) [38] (with support values ranging from 0.82 to 1) (Figure 2). In addition, these species showed high genetic distances from the *cytb* gene, ranging from 4.6% to 14.9% (Table 2).

Phylogenetic studies performed by Martínez-Ibarra et al. [10] and Martínez et al. [39] led those authors to propose changing the specific status of species *T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* to subspecies of *T. phyllosoma* (*T. p. bassolsae*, *T. p. longipennis*, *T. p. mazzottii*, *T. p. pallidipennis*, *T. p. phyllosoma* and *T. p. picturata*). However, the phylogenetic reconstruction obtained by combining different mitochondrial and nuclear markers enabled us to rescue the six species of the *T. phyllosoma* subcomplex as independent lineages (Figure 2), confirming the specific status of these vectors based on the phylogenetic concept of species (“... the smallest diagnosable cluster of individual organisms forming a monophyletic group within which there is a parental pattern of ancestry and descent” [40]).

Post-zygotic reproductive isolation barriers (sterility and/or hybrid collapse) that make the hybrids resulting from the crosses between *T. mazzotti* and most other species of the *T. phyllosoma* subcomplex unfeasible, as well as those between *T. phyllosoma* and *T. pallidipennis* and between *T. phyllosoma* and *T. bassolsae*, were described by Martínez-Ibarra [41–43] (Table 3). The characterization of these barriers under laboratory conditions confirmed the specific status of the parent species based on the biological species concept (“... groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups” [44,45]). Although the other experimental crosses did not allow the characterization of interspecific reproductive barriers (Table 3), these species showed high genetic distances from the *cytb* gene (the choice of the *cytb* gene to calculate the genetic distance was based on the minimum interspecific distance established by Monteiro et al. [46]), ranging from 4.6% to 14.9% (Table 2), which confirmed the specific status of all taxa, since these were greater than the minimum value established to separate species using the *cytb* gene (2%) [46].

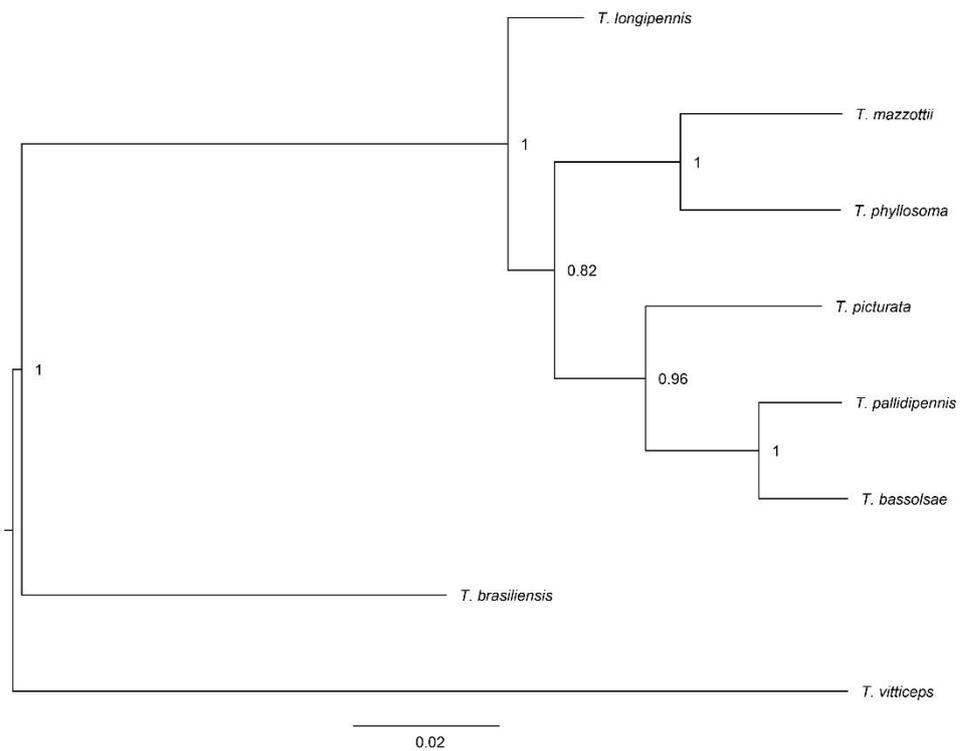


Figure 2. Bayesian phylogenetic tree. The posterior probability is indicated in the nodes.

Table 3. Experimental crosses carried out between species of the *phyllosoma* subcomplex.

Experimental Crosses	Pre-Zygotic Barriers	Post-Zygotic Barriers	References
♀ <i>T. mazzottii</i> × ♂ <i>T. longipennis</i>	Absent	Hybrid Collapse	Martínez-Ibarra et al. [41]
♂ <i>T. mazzottii</i> × ♀ <i>T. longipennis</i>	Absent	Hybrid Collapse	Martínez-Ibarra et al. [41]
♀ <i>T. mazzottii</i> × ♂ <i>T. picturata</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [41]
♂ <i>T. mazzottii</i> × ♀ <i>T. picturata</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [41]
♀ <i>T. mazzottii</i> × ♂ <i>T. pallidipennis</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [41]
♂ <i>T. mazzottii</i> × ♀ <i>T. pallidipennis</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [41]
♀ <i>T. mazzottii</i> × ♂ <i>T. bassolsae</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [41]
♀ <i>T. phyllosoma</i> × ♂ <i>T. pallidipennis</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [42]
♀ <i>T. pallidipennis</i> × ♂ <i>T. phyllosoma</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [42]
♀ <i>T. phyllosoma</i> × ♂ <i>T. bassolsae</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [42]
♀ <i>T. bassolsae</i> × ♂ <i>T. phyllosoma</i>	Absent	Hybrid sterility	Martínez-Ibarra et al. [42]
♀ <i>T. longipennis</i> × ♂ <i>T. picturata</i>	Absent	Absent	Martínez-Ibarra et al. [41]
♂ <i>T. longipennis</i> × ♀ <i>T. picturata</i>	Absent	Absent	Martínez-Ibarra et al. [41]
♀ <i>T. phyllosoma</i> × ♂ <i>T. longipennis</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. longipennis</i> × ♂ <i>T. phyllosoma</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. phyllosoma</i> × ♂ <i>T. picturata</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. picturata</i> × ♂ <i>T. phyllosoma</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. phyllosoma</i> × ♂ <i>T. mazzottii</i>	Absent	Absent	Martínez-Ibarra et al. [42]

Table 3. Cont.

Experimental Crosses	Pre-Zygotic Barriers	Post-Zygotic Barriers	References
♀ <i>T. mazzottii</i> × ♂ <i>T. phyllosoma</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. bassolsae</i> × ♂ <i>T. pallidipennis</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. pallidipennis</i> × ♂ <i>T. bassolsae</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. bassolsae</i> × ♂ <i>T. longipennis</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. longipennis</i> × ♂ <i>T. bassolsae</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. bassolsae</i> × ♂ <i>T. picturata</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. picturata</i> × ♂ <i>T. bassolsae</i>	Absent	Absent	Martínez-Ibarra et al. [42]
♀ <i>T. longipennis</i> × ♂ <i>T. pallidipennis</i>	Absent	Absent	Martínez-Ibarra et al. [43]
♀ <i>T. pallidipennis</i> × ♂ <i>T. longipennis</i>	Absent	Absent	Martínez-Ibarra et al. [43]
♀ <i>T. pallidipennis</i> × ♂ <i>T. picturata</i>	Absent	Absent	Martínez-Ibarra et al. [43]
♀ <i>T. picturata</i> × ♂ <i>T. pallidipennis</i>	Absent	Absent	Martínez-Ibarra et al. [43]

Usinger [15] was the first researcher to question the specific status of *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata*, suggesting the shifting of *T. phyllosoma* from a species to subspecies (considering this species as polytypic). Lent and Wygodinsky [47], based on morphological data, elevated its status to a species. On the other hand, Marcilla et al. [48], Martínez et al. [39], Martínez-Ibarra et al. [10] and Bargues et al. [49] performed molecular studies and observed very low interspecific variations, suggesting that classifying the species as subspecies would be more appropriate. However, Renfigo-Correa et al. [23,50], based on the phenetics and cohesion species concept considered *T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* as valid species. These concepts suggest, respectively, that "a species is a set of organisms that are phenotypically similar and that look different from other sets of organisms [51]" and "a species is an evolutionary lineage that serves as the arena of action of basic micro-evolutionary forces, such as gene flow—when applicable—genetic drift and natural selection [52]".

As mentioned above, although *T. longipennis*, *T. pallidipennis* and *T. picturata* live in sympatry and produce natural hybrids [10], there is some evolutionary factor that makes these hybrids unfeasible under natural conditions [which was not visualized under artificial conditions (Table 3)], since these taxa have a high interspecific genetic distance (Table 2), which demonstrates the genetic integrity of the three species, possibly resulting from reproductive isolation due to a post-zygotic barrier (a barrier that possibly inhibits the backcrossing and gene introgression between *T. longipennis*, *T. pallidipennis* and *T. picturata* under natural conditions).

Chagas disease is one of the most important yet neglected parasitic diseases in Mexico and is transmitted by Triatominae [53]. Nineteen of the 31 Mexican triatomine species are considered important species from an epidemiological point of view (including the six species studied here), as they invade human houses and all have been found to be naturally infected with *T. cruzi* [53]. The precise classification of *T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* species has epidemiological implications, as it allows vector control programs to direct monitoring and control activities directly to the species with the greatest vector importance.

These six species have interspecific morphological divergences that allow the species to be differentiated (also allowing the organization of dichotomous keys) [23,47]. Furthermore, the study of their external female genitalia [54] and the eggs [55] by means of scanning electron microscopy showed significant interspecific differences that allowed for the confirmation of the specific status of the species.

4. Conclusions

Thus, based on the literature data that were revisited and discussed here (morphological, genetic and evolutionary data), we confirmed the specific status of *T. bassolsae*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. phyllosoma* and *T. picturata* based on the phylogenetic, phenetic, cohesion and biological concepts of the species. Finally, we consider it important to carry out further studies to evaluate the presence/absence of interspecific gene flow (such as microsatellite markers and next-generation sequencing) between *T. phyllosoma* subcomplex species under natural conditions.

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