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**Evolución de la interacción *Trichobaris-Datura***

# T E S I S

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Me permito informar a usted, que el Subcomité de Biología Experimental y Biomedicina, en su sesión ordinaria del día 20 de junio de 2016, aprobó el jurado para la presentación de su examen para obtener el grado de **DOCTORA EN CIENCIAS**, del Posgrado en Ciencias Biológicas, de la alumna **DE LA MORA CURIEL MARISOL** con número de cuenta **302086537** con la tesis titulada: **"EVOLUCIÓN DE LA INTERACCIÓN *Trichobaris-Datura*"**, bajo la dirección del **DR. JUAN SERVANDO NÚÑEZ FARFÁN:**

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Sin otro particular, me es grato enviarle un cordial saludo.

**A T E N T A M E N T E**
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ÍNDICE

**RESUMEN**

Los curculiónidos (Curculionidae) son un grupo de coleópteros extraordinariamente diverso y asociado con las plantas, principalmente angiospermas. El desarrollo larvario de estos ocurre dentro de los tejidos de las plantas. Puesto que la tasa de diversificación entre plantas y curculiónidos está desfasada, son necesarios estudios a un nivel taxonómico menor para entender cómo las plantas han influido la diversificación de los escarabajos. Se espera que el tipo de interacción y el grado de especificidad afecten los patrones de variación genética entre curculiónidos y sus plantas huésped; supuestamente la especialización de los insectos en el uso de una planta huésped promueve una reducción en la variación genética de los escarabajos ya que se vuelven más eficaces en el uso de la planta, y no requieren mantener altos niveles de variación genética. El objetivo de esta investigación es el análisis de la variación genética de los curculionidos del género *Trichobaris* que difieren en el grado de especificidad respecto de su planta huésped. Para ello se hizo el análisis filogeográfico de dos especies del género, una especialista y otra generalista, así como el análisis filogenético del género y la estimación de la planta huésped ancestral. Para el primer objetivo, encontré que no existe una reducción en la variación genética de *T. soror*, un especialista, en comparación con un generalista *T. compacta*. Respecto a la estructuración poblacional: *T. soror* presenta dos grupos genéticos a lo largo de su distribución geográfica, mientras que para *T. compacta* cuatro grupos, uno de ellos ampliamente distribuido. La filogenia del género muestra, por primera vez, las relaciones evolutivas entre especies de *Trichobaris*, y sugiere convergencias en morfología y morfotipos para una misma especie. La reconstrucción de la planta ancestral indica que las interacciones conservadas serían aquellas entre *Trichobaris soror-Datura stramonium*, *T. compacta-D. wrightii* y *T. texana-Solanum eleagnifolium*. La presencia de *Trichobaris* spp. en otras especies de huésped sugiere que se trata de colonizaciones recientes; tal es el caso de las plantas cultivadas *Solanum tuberosum* (papa) y *Physalis ixocarpa* (tomate).

**ABSTRACT**

Weevils (Curculionidae) are an extraordinarily diverse group of beetles (Coleoptera) associated with plants, mainly angiosperms, whose larval development occurs inside the tissues of these plants. Diversification rates of both groups show temporal lags. Thus, studies at lower taxonomic level are needed to better understand how host plants influence the diversity of weevils. It is expected that both the type and degree of specificity of interactions impact the patterns of genetic variation among weevils and their host plants. Host specialization would promote reductions of genetic variation in beetles as they become more efficient in host use. The aim of this research is to analyze the genetic variation of weevils in the genus *Trichobaris* that vary in their specificity to the host plant. For this, I analyzed the phylogeography of two species, the host specialist *Trichobaris soror*, and the generalist *T. compacta*. Then, I obtained the phylogeny of the whole genus *Trichobaris*, and aimed to reconstruct the ancestral host plant. I found that both species show similar levels of variation. In *T. soror* I detected two genetic groups along its geographic distribution whereas in *T. compacta* I found four groups, one of them widely distributed. The phylogeny of *Trichobaris* shows, for the first time, the evolutionary relationship between species of *Trichobaris*, and suggest convergence in morphology and morphotypes for the same species. The reconstruction of the ancestral host plant suggests conserved interactions between *T. soror-Datura stramonium*, and between *T. compacta-D. wrightii* and *T. texana-Solanum eleagnifolium*. The interaction between *Trichobaris* and cultivated host plants, *Solanum tuberosum* (potato plants) and *Physalis ixocarpa* (tomato plants), seems to represent a recent colonization.

## INTRODUCCIÓN GENERAL

En la presente tesis de investigación se estudió el género *Trichobaris* (Coleóptera: Curculionidae) tanto a nivel filogeográfico como filogenético para inferir cómo la interacción con distintas especies de planta huésped pudo haber repercutido en su diversidad. La característica principal de este tipo de interacción entre insectos y plantas recae en el estudio del proceso de especiación, sin embargo éste es un proceso muy complejo, por lo que a continuación se presentan tres capítulos de revisión dónde se señalan los alcances y limitaciones en el estudio de este tipo interacción entre insectos y plantas.

El proceso de especiación ha demostrado ser un proceso difícil de observar, por lo que un enfoque filogeográfico combinado con uno filogenético pueden darnos información relevante que guíe el estudio de la especiación en un grupo particular. En el capítulo 1 de esta tesis se describen los modelos de especiación que se han propuesto y las inferencias que pueden hacerse a partir de ellos, seguidos de la interpretación de los patrones filogeográficos y filogenéticos. El tipo de información filogeográfica va desde la descripción de la influencia de factores microevolutivos en la estructuración geográfica y discontinuidades genéticas a lo largo de la distribución de las especies, hasta la información filogenética que estima las relaciones evolutivas entre las especies y da lugar a las hipótesis ecológicas para la inferencia de las presiones selectivas que dieron lugar a las especies del grupo.

La especiación en particular de los insectos herbívoros se ha explicado por asociación a las plantas con las que interactúan, constituyendo éstas una presión de selección. En el capítulo 2 se discute y ejemplifica como la naturaleza de la interacción, es decir el tipo de interacción y el grupo taxonómico evolutivo al que pertenecen los interactuantes (insectos y plantas), debe ser considerado en el estudio de la especiación de insectos herbívoros para poder encontrar tendencias evolutivas a nivel micro-evolutivo y macro-evolutivo.

Los escarabajos curculiónidos son una de las familias de coleópteros con más especies descritas conocidas. El éxito evolutivo del grupo se ha asociado con la diversificación de las angiospermas, a pesar de que otros factores clave favorecieron su diversidad y abundancia. En el capítulo 3 se hace

una revisión de la evolución de este grupo en la que se reconoce que las plantas constituyen una importante presión selectiva para este grupo por lo que conocer cómo están moldeando la diversidad genética de los curculiónidos es primordial para entender su proceso de especiación.

El género *Trichobaris* es un grupo de curculiónidos descritos por Barber en 1935, a la fecha no hay una hipótesis ecológica que explique su diversidad ni una hipótesis filogenética que establezca las relaciones evolutivas entre sus especies. En el sistema de estudio y el planteamiento del problema se detallan los antecedentes del género y la pregunta que inspiró esta tesis. En seguida de la hipótesis y los objetivos se presentan los artículos resultado de este trabajo de investigación. Finalmente se discuten de marea general los resultados y se presenta las conclusiones.

## Capítulo 1

**Phylogeographic and phylogenetic patterns of speciation.**

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This review focuses on the assumptions and predictions of different speciation models with a brief critique, and how we can interpret phylogeographic and phylogenetic patterns in order to suggest the evolutionary forces that have been operating to promote genetic differentiation and sometimes speciation under the model of speciation by selection.

Speciation is one of the most important problems in evolutionary biology, where many controversies remain (Sobel et al. 2010). The term “speciation” is applied to several processes that involve the production of new evolutionary lineages (species) (Wiley, 1981). This review assumes the species definition given by Simpson (1951), namely a population system which possess the following characteristics: (1) It is a lineage, an ancestral-descendant sequence of populations in space and time. (2) The lineage evolves separately from other lineages, in other words from other species. (3) It has its own unitary evolutionary role, that is it fits into its own particular ecological niche in a biotic community. (4) And it has its evolutionary trends, being susceptible to change its evolutionary role during the course of its history.

Some models have been proposed in order to explain the speciation process and at the same time to make predictions (Box 1) (Coyne and Orr, 2004; Morrone, 2013; Nosil, 2012, Wiley, 1981; 2011). The genetic models include: speciation due to polyploidy, hybridization and mutation-order speciation (Coyne and Orr, 2004; Nosil, 2012; Mendelson, et. al. 2014). Geographic models include: allopatric, peripatric, parapatric and sympatric speciation (Coyne and Orr, 2004; Wiley, 1981; 2011). Finally, the model of ecological speciation, at difference of the others, explicitly deals with the force that promotes speciation (Nosil, 2012).

The classical models of speciation by polyploidy, hybridization, mutation-order speciation, and others, describe the genetic processes of genetic differentiation but these phenomena do not by themselves promote speciation. We can see the genetics of speciation as the study of the genetic basis of characters that are directly associated with ecological interactions and adaptation (Via, 2002). But it can be descriptive too about how the gene pool of a species becames different and increases reproductive isolation, including patterns of divergence that persist as long as evolutionary distance increases such, as genomic islands of divergence, genetic hitchhiking, accumulation of neutral mutations and outlier loci (Nosil & Feder, 2012; Supple et al., 2014). Even so, polyploidy, hybridization, and mutation-order speciation can be classified as patterns of genetic divergence as a results, some authors argue, to the fact that that natural selection is a ubiquitous part of speciation and assume that polyploidy speciation and mutation-order speciation are often cases of ecological speciation (White, 1978; Sobel et al., 2010).

Surely, genetic, geographic and ecological models are not mutually exclusive. A way to integrate these models is considering the evolutionary factors of population genetics. If we think of species as a single lineages, what factors cause population differentiation until reproductive isolation arises and thus the independence of lineages establishes? Essentially the same factors that generate linkage disequilibrium: mutation, drift, migration and selection (Otter & Endler, 1989). In fact, the definition of geographic models of speciation implies some gene flow among populations but the emphasis is given to geographic distribution (Wiley, 1981) and not to the force that is promoting speciation. Gavrilets (2003; 2004) mentions that speciation can be understood as divergence along nearly neutral networks and holey adaptive landscapes (driven by mutation, drift and selection for adaptation to a local biotic and/or abiotic environment) accompanied by the accumulation of reproductive isolation as a by-product. The main assumption here is that population differentiation leads to lineage independence.

The model of speciation according to these evolutionary factors can integrate the genetic and geographic models in order to explain in a more complete way the speciation process more complete. Speciation by drift events means that speciation occurs in any particular direction because the conditions that led it (e.g. high mutation rate and small population size). Migration is supposed to reduce as speciation takes place (some considerations about the genomic architecture in divergence with or without gene flow is reviewed by Feder et. al. 2013). Speciation by selection

indicates there is a selection pressure that is promoting differentiation. It can be divided in ecological (Nosil, 2012) and sexual selection but not exclusively. Certainly, the source of selection can potentially be differences between abiotic factors in the environment, distinct interactions between populations and other species, and a combination of these different sources. In fact, some studies point the influence of more than one selective pressure to achieve reproductive isolation (Berlocher & Feder, 2002).

All evolutionary factors described by population genetics participate in the speciation process but the point is to identify how they work together, clarifying which are the speciation promoters (selective factors), the participation of drift (by means of historical demography and effective population size) and migration (measuring gene flow) and describing how genetic differentiation is being achieved due to mutations, hybridization, polyploidy and so on. In this approach speciation parameters depend on evolutionary factors (Gavrilets, 2003) and not on species distribution.

The expected time to speciation, led by mutation and drift is typically very long. However, selection for local adaptation can significantly decrease the time of speciation. Theoretical studies predict extreme sensitivity to the likelihood of speciation and the waiting time for speciation in the model parameters, which in turn rely heavily rely on environmental conditions thus suggesting that overall speciation is triggered by changes in the environment Gavrilets (2003).

Two fundamental issues can limit the predictions in speciation: the genetic diferentiation through mutation and the phylogenetic-ecological restrictions. Under this approach the evolutionary factor “mutation” that describe how genetic variation arises should be replaced by genetic processes that shape genetic differentiation, we should say that describes what is going within the gene pool of a species while speciation comes about (and of course, describe how genetic variation is arising in the population gene pool). The phylogenetic-ecological constrains can be very important in the theory of speciation because we are not starting from the very beginning, and the “how” organisms are responding to selection is an other knowledge that can give us the evolutionary trends in speciation (Gilbert, 2015).

The use of just genetic variation limits our predictions, even so, the practice of interpreting genetic variation with the idea of speciation by selection and speciation by drift events (as a null model) can help us make some assumptions and predictions of genetic variation over the phylogeographic and phylogenetic studies (Figure 1). Here I show some patterns in phylogeography and phylogeny that can be viewed under this approach to speciation, having two temporal windows looking back in speciation. An extended practice is to use microsatellite, DNA sequences from mitochondria and chloroplast, and DNA sequence from nuclear genes.

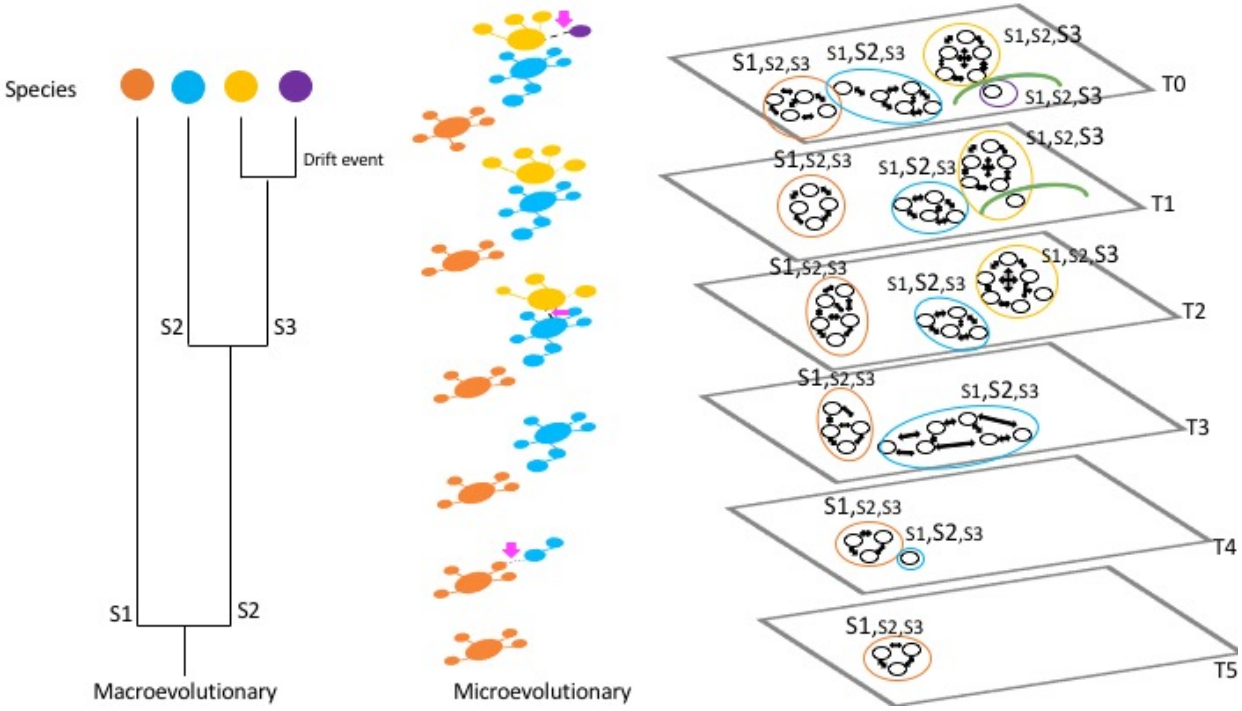


Figure 1. Speciation at macroevolutionary and microevolutionary scales under the selection model of speciation. On the right, several sources of selection (S1, S2 and S3) promoting population differentiation at different times and space. Small white circles represent populations and the black arrows among them, genetic flow. Populations of the same species are surrounded by colored circles. A stochastic event is marked with a green line on time T1 and T0. In the middle, microevolutionary view of species haplotypes networks, the initial breaks on genetic pools are indicated by a dashed line and a pink arrow. On the left, microevolutionary view of species phylogeny indicating species raised by a constant selective pressure of selection and the repercussion of the drift event on speciation.

In the phylogeographic approach (microsatellites, DNA sequences from mitochondria and chloroplast data, usually included) a first point is the recognition of geographic distribution and spatial genetic structure of populations within a species. At this point the study of speciation is at

the microevolutionary level. The observation that variation can often cut across species boundaries indicates that an investigator could pursue the elucidation of the causal agents responsible for spatial patterns of phenotypic or genotypic variation, and in second place, to identify microevolutionary factors that are promoting speciation (Otte & Endler, 1989). Commonly microsatellite, DNA sequence from mitochondria and chloroplast, and DNA sequences from nuclear genes vary on mutation rate and the degree of homoplasy they show (Roderic, 1999; Chung-Ping & Danforth, 2004) making inferences at different times in lineage evolution possible.

**Speciation at the microevolutionary level**

With microsatellite and DNA sequences (from mitochondria and chloroplast) data we can infer genetic diversity, clustering and genetic breaks, local adaptation (and coevolution), rates of gene flow (m), historical demography and genealogical relationships among populations. With DNA sequence from nuclear data (highly conserved genes and with a low recombination rate) we can infer: phylogenetic relationships, adaptive radiations and historical relationships as: colonization, cospeciation and coevolution.

Genetic diversity

Estimates from DNA sequences: like heterozygosity (*H*), haplotype and nuclear diversity (*h* and $\pi$) or the number of segregating sites (*S*) are strictly linked to demographic history (Avise, 2000). A population with low *h* and low $\pi$ may have experienced a prolonged or severe demographic bottlenecks (or, perhaps, a selective sweep) in recent times. Conversely, high values for *h* and $\pi$ are an expected signature for a stable population with large long term *Ne*; or they also might be observed in an admixed sample of individuals from historically partitioned populations. High *h* and low $\pi$ suggest rapid population growth from an ancestral population with small *Ne*. Low *h* and high $\pi$ could result from a transient bottleneck in a large ancestral population; also they can reflect an admixture of samples for small, geographically subdivided populations (Avise, 2000).

Clustering and genetic breaks

The genetic breaks are defined as a shallow or deep discontinuities (depending on the genetic distance) in the genetic variation throughout the species geographic distribution. Gavrilets (2003) notes that a wide geographical distribution can generate the initial genetic break between

populations of a species, forming small founder populations outside the distribution range of the species and / or separated in distinct microhabitats. In DNA sequences, Avise (2004) describes the interpretation of the genetic breaks as two aspects of the genealogical concordance : (1) any deep phylogeographic split deduced in a gene genealogy is given by multiple independent changes on the molecular sequence (which on a haplotype network can be seen as deep or shallow genetic break), (2) the phylogeographic breaks in a gene tree can arise not only from a long term vicariance separation but also from isolation by distance in continuously distributed species. The genetic breaks also can be explained by strong divergent selection (Marske, et al. 2013)

To distinguish the gene-idosyncratic or spatially haphazard genealogical breaks (due to isolation by distance, or perhaps to gene-specific selection) from ancient vicariance-induced genealogical breaks (whose effects are more likely to be genomically extensive), a congruence between different genes should be expected (Avise, 2004).

Local adaptation

Here the common measure is population differentiation (*Fst)* in order to look for associations due to environmental factors or other species which one interacts, and probably have a reciprocal influence (coevolution). This measure can be influenced by the number of loci involved in the trait that is under selection, the degree of genetic hitchhiking and the amount of gene flow that still occurs among populations. Some alternatives are to look for individual loci under selection (Beaumont, 2005). With DNA sequences, local adaptation is looked through correspondence between genealogies or associations of haplotypes with respect to an environmental factor or interspecies interactions. Both describe an alternative explanation of the genealogical concordance (see above).

Rates of gene flow

Migration among populations is directly related to population differentiation; Nosil (2012) mentions that at early states that the amount of gene flow will be decrease until speciation arises. The gene flow estimated in “recent history” can be mistaken with the homoplasy of the markers (e.g. microsatellites). It has been suggested on the other hand that *Fst* estimates are not reliable when some assumptions are violated (Whitlock & Mccauley, 1999). Rates of gene flow, measured through DNA sequences can be hardly influenced by incomplete lineage sorting at early differentiation. Recent gene flow will reduce differentiation and increase the proportion of shared

alleles among closer populations as compared to distant populations (Nosil, 2012). Also, gene flow should initially result in strong linkage disequilibrium between shared alleles at linked sites, which would breakdown over time if gene flow goes on for longer times periods. Small amounts of gene flow between populations spurs population differentiation; conversely, migration can significantly delay speciation (Gavrilets, 2003).

Here, it is relevant to check for *incomplete lineage sorting* that is the retention and stochastic sorting of ancestral polymorphism, the genealogical histories of individual gene loci may appear misleading or uninformative about the relationships among species or populations (Maddison & Knowles, 2005). This is especially likely if the widths of lineages (i.e., the effective population sizes, *Ne*) are large relative to their lengths (i.e., the time between divergences). In this case, genetic drift is unlikely to have time to bring loci to fixation before subsequent divergences (Pamilo & Nei, 1988). Although increasing the number of loci gives more accurate trees for a given sampling effort with deeper species trees, sampling more individuals often gives better results than sampling more loci with shallow species trees (Maddison & Knwoles, 2005).

Historical demography

*Ne* and *Tau* estimations of the historical demography in order to know effective population size can be done through pairwise comparisons, assuming that the mutation rate is known. Mitochondrial DNA haplotypes can be very informative to check the genetic structure in a particular species. Of course these patterns should be confirmed with several genes or others genetics markers in order to have a reliable estimation of the genetic structure (Avise, 2004; e.g. Vences, et al. 2013).

Haplotype patterns: The “star-like” pattern is found in cases where there is high haplotype diversity. In this case most of the haplotypes occur at very low frequencies and have low differentiation from the more frequent (probably ancestral) haplotype. This pattern is indicative of sustained increase in population size possibly because a species is expanding its geographic range for the first time or went through a bottleneck event followed by population expansion (Slatkin and Hudson, 1991; Allcock & Strugnell, 2012). The “disjunct” statistical parsimony haplotype network that is fragmented with multiple networks with very low haplotype diversity appears to be the result of small isolated populations that underwent bottlenecks, where genetic drift had a much more pronounced effect on these reduced gene pool than it would have in larger, more genetically diverse populations. Here, selection leads to rapid fixation so it is possible also to find an effect of selection

on this pattern (Allcock and Strugnell, 2012). The “parodical” haplotype network has a large number of haplotypes but most are restricted (e.g. to a geographic locality), this reflecting local diversification and poor dispersal. At the same time, it shows that there has not been a major bottleneck because high haplotype diversity is maintained. The “diffuse” haplotype network displays a high haplotype diversity (Allcock & Strugnell, 2012), and it can have a combination of the parodical and star-like patterns which are indicative of a complicated demographic history with less dramatic reduction in haplotype diversity and probably extension of its distributional range.

There are some general conclusions about genealogical concordance at the process of divergence, that Avise (2000) named the third and four aspects: the superspecies level of genealogical concordance, explains the biodiversity patterns (Marske, et al. 2013). The congruence in the phylogeographic pattern of another species in the same area, implies that the biota distributed in that region responded similarly to climatic or geological history of their current range distribution; this means that phylogeographic partitions that are similar in spatial placement and perhaps in temporal depth. To conclude we should expect concordance between molecular genetic data and traditional biogeographic evidence based on non-molecular data (Avise, 2006). The genealogical discordance between the genealogy of a species and its distribution suggests that is not generalizable that a combination of historical vicariance and contemporary selection explain the speciation for a species.

**Speciation at macroevolutionary level**

With nuclear DNA sequence data, we can infer phylogenetic relationships, adaptive radiations, the historicity of the interaction: colonization, coespeciation and coevolution (Pagel, 2003).

Phylogenetic relationships

Species-level phylogenies derived from molecular data provide an indirect record of the speciation events that have led to extant species. We should ideally sample all the species in a higher group, such as a genus, ensuring that those species reflect evolutionary entities within the group and rule out the effects of other processes as good explanations for observed patterns (Barraclough and Nee, 2001). Avise (1999) proposes that the approximate dates of nodes in evolutionary trees should be the universal criterion according to which taxonomic classifications above the level of biological species are erected. Some authors are claimed to do the phylogeny at genus level in order to discover the aspects that promote speciation.

Historical relationships

Colonization is when speciation occurs previously to settlement in a new area. For this it is necessary to have the phylogeny calibrated and the time of formation of the area (e.g. Percy *et al*., 2004). As I mentioned before a source of selection can potentially be the interaction with other species; a way to explore "historical associations" is the comparison of species trees (Page, 2003). This means that we need to look for coespeciation, when two groups speciate together in space and time (Cruaud *et al*., 2012). Under coespeciation we can find some macroevolutionary events that influence the phylogenetic congruence particularly in the case of parasites and host. Page (2003) identified the following: host switching (a duplication event, one new lineage moves to a different host lineage), duplication (the associate speciates independently of the host) and "missing the boat" (a kind of lineage sorting where at a divergence point in the host phylogeny, the associated lineage does not apparently diverge). If during cospeciation species have imposed selection pressures reciprocally we can say they have coevolved (Berlocher, 2000).

Adaptive radiation

The adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage. It involves the differentiation of a single ancestor into an array of species that inhabit a variety of environments and that differ in morphological and physiological traits used to exploit those environments (Schulter, 2000). We can detect adaptive radiations when we observe a fast diversification process on a phylogeny followed by ecological and phenotypic divergence (Schulter, 2000). Here, we can corroborate the four criteria that defined it: a common ancestry of the component species, phenotype-environment correlation, trait utility and rapid speciation (Schulter, 2000, Glor, 2010). Glor (2010) summarized these two components in a single criterion called adaptation. In practice, the term adaptive radiation is applied at low and high taxonomic levels however it is more accurate to apply it at the level of closely related species within the same genus because the mechanisms of divergence are clearest at this level (Schulter, 2000).

As a conclusion genetic variation can give insights into the speciation process with phylogeography patterns at early stages in population differentiation influenced by drift and gene flow and promoted by selection. Phylogenetic patterns show the products of the speciation process and are very useful to infer the role of selective pressures that originated them. As each species or group of species have

its own evolutionary tendencies our predictions in speciation are limited until the genetic processes that shape the divergence and how are species responding to selection forces, it must be considered into account in studies of speciation.

Box1. Speciation models

| **Speciation models (definition)** | **Assumptions** | **Predictions** |
|---|---|---|
| **Genetic Models** | | |
| **Polyploid speciation**<br>New species are originated due to a numerical change in a whole set of chromosomes. | 1.- No so many genetic restrictions.<br>2.- New polyploids will be isolated from parental species due to disjunctions in the meiosis. | 1.- Individuals will continue doubling its stable chromosome number, each generation.<br>2.- Reproductive isolation is instantaneous. |
| **Hybrid speciation**<br>New species are originated due to the crossing of two parental species. | 1.- Genetic compatibility<br>2.-It occurs within the distribution of parental species<br>3.- There is not selection against hybrid genotypes on the contrary hybrid genotypes can confer ecological advantage.<br>4.- Assortative mating in parental and hybrid species. | 1.- The genome of the new species is a mosaic of pieces from the parental species<br>2.- Hybrids will be observed between the distribution of parental species.<br>3.- Hybrid genotypes that confer an ecological advantage and influence assortative mating could quickly result in the origin of a novel hybrid population that is reproductively isolated from the parental species. |
| **Mutation-order speciation**<br>New species are originated due to separate populations adapting to similar selection pressures fix different advantageous mutations (alleles) that are incompatible with one another. | Selection arising from sexual or genetic conflict. | Reproductive isolation is uncorrelated with ecological divergence and correlated with the intensity of conflict. |
| **Geographic Distribution Models** | | |
| **Allopatric**<br>Lineage independence is achieved while two or more lineages are geographically | 1.-Demes tend to differentiate (within the limits imposed by developmental homeostasis) in response to stochastic and local | 1.-Given an initial amount of geographic variation, disjunction is likely to result in differentiation because gene flow ceases across the disjunction.<br>2.-The relative apomorphy or plesiomorphy of a particular daughter species for a character that is polymorphic in the ancestral population and varies |

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| disjunctive (separated, vicariate). | extrinsic factors (i.e. selection).<br>2.-Deme differentiation is countered by the genetic flow within the species range.<br>3.-Between-deme differences are inversely proportional to interdemic gene flow and population size and directly proportional to selective differences between demes, the rate of origin of unique evolutionary innovations , and the initial geographic variation of the ancestral species (Wiley 1981) | geographically will be determined by geography. Time to fixation will be determined by number and size of the demes comprising the daughter species and the selection differences among them.<br>3.-The relative apomorphy or plesiomorphy of a particular daughter species for an evolutionary novelty after disjunction cannot be predicted and therefore can be assumed to be random.<br>4.-Time to differentiation may be relatively long and will depend on interdemic migration, deme size, the number of demes in the smaller of the two daughter species, and the selective advantage of the different characters.<br>5.-A phylogenetic hypothesis of the relationships of the species (as evidenced by synapormorphies) should reflect accurately the temporal geographic separation (vicariance) and speciation of these species.<br>6.-The range of the ancestral species may be estimated by adding the ranges of the daughter species.This prediction should hold for relatively young speciation events and for older events if the daughter species are poor dispersers.<br>7.-The geographic point of disjunction corresponds to boundary between disjunct or continuous daughter species.<br>8.-We should expect to see many clades that inhabit the same geographic area to show the same (congruent) phylogenetic and biogeographic patterns (Wiley 1981). |
| **Peripatric**<br>New species arises in marginal, habitats, usually in the boundary of a larger central population. | 1.-Demes tend to differentiate (within the limits of developmental homeostasis) stochastically and in response to local factors of the environment (selection) but interdemic migration is prevalent enough within the more central parts of the species' range to prevent differentiation of any one or any combinations of demes.<br>2.- Interdemic gene flow is not strong enough at the periphery of a species boundary to prevent the establishment of new | 1.- New species by appear initially in marginal habitats, often but no means always, at the margins of the ancestral species' range. The place of the appearance is causally related to a geographic disjunction which prevents interdemic migration. The original range of the new species will be small relative to the ancestor.<br>2.- The peripherial isolate will be more apomorphic than the central population regardless of whether the central population becomes a species different from the ancestral species. Further, the distribution of apomorphic characters cannot necessarily from the geographic variation of the ancestor.<br>3.- The divergence of the peripherial isolate will be random with respect to the ancestral array of epiphenotypes within the constrains of developmental homeostasis.<br>4.- If the history of speciation of a group involved peripherial isolation, speciation, subsequent migration of the peripherial isolate, and other |

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| | phenotypes at relatively high frequencies. Further, if interdemic migration is stopped (i.e. disjunction occurs), one or more of these novel phenotypes may become fixed as a new species (Wiley, 1981). | peripherial isolation and so on, such that progression in time and space is the result, then we might expect the pattern of descent as evidenced by synapomorphies to be largely dichotomous.<br>5.- If the history of a group involved peripheral isolation and speciation of a number of peripherial isolates around the range of a single ancestral one, then we should expect the pattern of descent as evidenced by synapomorphies to be polytomus (a multiple furcation) because the descendant species would share with each other only the common characters also shared by the ancestral species and all other peripherial isolates.<br>6.-If the assumption inherent to prediction 4 applied, the dispersal through time and space must produce a pattern of biogeography concordant in every respect with the phylogenetic relationships of the species group.<br>7.- If the assumption inherent to prediction 5 are apply, then dispersal through time and space should not follow either the deviation rule or the progression rule (that is, one daughter species must be primitive in morphology and occupy the ancestral range whereas the other daughter species must be more derived (Henning's deviation rule), and occupy newly gained geographic space or its original range (Henning's progression rule)).<br>8.- Due to different dispersal capabilities, the biogeographic patterns of different species groups inhabiting the same geographic range will not be expected to show similar biogeographic patterns under the assumptions inherent to either predictions 5 and 6 (Wiley, 1981). |
| **Sympatric**<br>Lineage independence is achieved without geographic separation by shifts in ecology, host, or timing of reproduction, or by hybridization or apomixis. | No geographic segregation. | No predictions in geographic terms. |

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| **Parapatric**<br>Lineage independence is archived between geographically distinct lineages with maintain limited interlineage mating across a contact zone. | 1- Demes tend to differentiate in response to stochastic processes and local selection within the limits of developmental homeostasis.<br>2.- Deme differentiation tends to be countered by interdemic migration within the range of the ancestal species.<br>3.- The individual members of the ancestral species have relatively low vagility and thus local differentiation is pronounced.<br>4.- Given a decrease in fitness of a heterozygous epiphenotype along a geographic variation gradient (a cline), assortative mating may occur such that speciation may go to completion (Wiley, 1981). | 1.- If there is competition among the new sister species, then a narrow zone of sympatry will be established. Due to interdemic migration, we would expect this situation given that one daughter species does not outcompete and thus eliminate the other.<br>2.- The phylogenetic pattern of parapatric speciation as evidenced by apomorphic characters is similar to that expected from vicariance allopatric speciation; that is, we would expect a mostly dichotomous pattern.<br><br>3.- The relative apomorphy or plesiomorphy of any one of the daughter species will be determined, assuming no evolutionary innovations, by the geographic distribution of characters in the ancestral species. The origin of any particular novelty after establishment of the contact zone (effectively after the onset of speciation) cannot be predicted (Wiley, 1981). |
| **Ecological speciation**<br>Barriers to gene flow evolve between populations as a result of ecologically based divergent selection between environments. | 1.- There is a source of divergent selection.<br>2.- There is a form of reproductive isolation.<br>3.- A genetic mechanism link selection to reproductive isolation. | 1.- Speciation events require ecological shifts.<br>2.- Levels of reproductive isolation are positively correlated with levels of ecological divergence between populations pairs, independent of time.<br>3.- Traits under divergent selection may also affect reproductive isolation.<br>4.- Divergent selection results in ecological selection against immigrants and hybrids.<br>5.- Adaptive divergence reduces gene flow between populations. |
| **Evolutionary Force Models** | | |
| **Speciation by drift events**<br>New species arise as a consequence of stochastic and random events acting on populations. | 1.- Any selection pressure.<br>Sets geographically structured populations with few migration among populations.<br>2.- Small effective population size due to founder effect or “bottleneck”. | 1.- Speciation events require drift events.<br>2.- Population differentiation is due to lack of genetic flow, inbreeding and random genetic variation.<br>3.- Reproductive isolation is correlated with the occurrence of population bottlenecks, perhaps also time.<br>4.- The genetic breaks along species distribution should be coincident with barriers to gene flow.<br>5.- These barriers can be geographical (Harrison, 1999) or temporal (e.g. different time to bloom). |

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| | 3.- The genetic processes that shape genetic differentiation is random. | 6.- It is impossible to predict evolutionary trends in population differentiation. |
| **Speciation by selection**<br>New species arise in respond to selective forces acting on populations. | 1.- Several sources of selection can coexist: abiotic factors and interactions with other species, which promote population differentiation.<br>2.- Some sources of selection are leading more directionality that others on differentiation among populations.<br>3.-Geographically structured populations where gene flow counters population differentiation.<br>4.- Population size is large enough to prevent drift.<br>5.- The genetic processes that originate genetic variation and the processes responding to selection depend on the nature of the lineage. | 1.- Speciation events require selection pressure.<br>2.- Among several sources of selection, population differentiation will be correlated in a proportional fashion with the intensity of each one.<br>3.- Selection sources deplete or decrease gene flow which brings about population differentiation.<br>The genetic breaks along species distribution should be coincident with selection sources.<br>4.- Genetic drift does not influence population differentiation.<br>5.- Given that speciation events require selection pressure, the speciation events in phylogenetic trees are accompanied by ecological change.<br>6.- Genetic processes responding to selection will lead the evolutionary trends in population differentiation. |

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**Insect speciation and plant interaction**

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My aim here is to point out the working hypothesis in the study of speciation of insects associated with plants, emphasizing its role as a selective pressure but at the same time pointing out that it is not the only source of selection. Also to stress the importance of evolutionary lineages trends that are being studied to find patterns that explain the diversity of insect species.

Insects are one of the most diversified lineages (Grimaldi, 2005). The diversity of phytophagous insects is strongly associated to the rise of flowering plants. There is no doubt that the diversification of plants (mainly angiosperms) affected the diversity of insects (MacKenna, 2009; Janz, 2011; Grimaldi, 2005). Winkler and Miller (in Tilmon, 2008) made a review of the events of speciation accompanied by change in host plants (based on 45 phylogenies of phytophagous insects for which information on host plants and their range was available) and found that about 48% of divergence events were associated with an apparent change in the host plant. This association can not be due to chance.

The evolutionary hypothesis that supports the idea of speciation of insects associated to a host plant holds that:

*Angiosperms have through occasional mutations and recombination, produced a series of chemical compounds […], these compounds, by chance, serve to reduce or destroy the palatability of the plant […], such plant protected from the attacks of phytophagous animals, would in a sense have entered in a new adaptive zone. Evolutionary radiation of the plant might follow, and eventually what began as a chance mutation or recombination might characterize an entire family or group of related families. […] If a recombinant or mutation appeared in a population of insects that enabled the individuals to feed on some previously protected plant group, selection could carry the line into a new adaptive zone. Here it would be free to diversify largely in the absence of competition from others phytophagous animals* (Ehrich & Raven, 1964).

This hypothesis in based on the main assumption that specialization on plants brings insect speciation and departs from the microevolutionary level to explain the macroevolutionary level, which give us a framework to study insect-plant interactions. Although other hypotheses have been proposed as Oscillation of Host Plant Range and Speciation (Janz and Nylin, on Tilmon, 2008) much work is still need to integrate a framework of speciation in insects associated to plants through specialization (Forister et al. 2012). We need a careful examination of the trends in insect and plant lineages, to identify which one is the selection pressure to respond at, and the intensity with which it acts on insects.

The distribution of herbivore diversity across plant taxa may be highly unbalanced in part for historical reasons because not all insect clades restricted to a host are comparable in age to their host lineages, and some traits may form coadapted strategies. Thus an important question is whether these defenses are against a narrow or a wide range of herbivores, and the historicity of the interaction (Futuyma & Agrawal, 2009).

Insects, like other organisms, are influenced by micro-evolutionary factors: mutation, drift, migration and selection. And selection in this scenario is commonly related to a host plant preference, and placed in the model of ecological speciation (Nosil, 2012;). Here I want to point out that even if the insect interacts with a particular plant, the selective agent may vary, and not in all cases this interaction translates as coevolution (Janzen, 1980; Janz, 2011). Different sources of selection can be promoting speciation, apart from plant preference, such as predation (cryptic morphology), mating preference, intraspecific competition etc. (e.g. Supple 2014; Matsubayashi et al., 2010). And speciation via random genetic drift, is likely through founder events and population bottlenecks, especially on insects with patchy distributions and small local population sizes caused by host-plant shifts, or because they that have recently colonized islands and do not possess high dispersal abilities (Matsubayashi et al., 2010; Roderick, 1996; Roderick and Gillespie, 1998).

As a consequence, genetic variation between specialist and generalist insects can result from selective pressures that the plants impose in a few or many loci that are responding to it, as well as other selective agents, in addition to the role of geography and historicity of species interactions.

In the study of insect divergence and plant associations might be worth considering some points of this interaction with the aim of reaching an integrative view of insect speciation and discern some trends on insect evolution:

At the ecological level.
1) The nature of the interaction.
At the microevolutionary level.
2) Characteristics that mediate the interaction and have an effect on local adaptation.
3) A form of reproductive isolation that links selection with genetic differentiation.
4) Geographic mosaic of coevolution.
5) Intraspecific phylogeography.
At macroevolutionary level.
6) Interspecific phylogeography.
7) Conservatism of interactions: coevolution, cospeciation and colonization.
8) Mapping key traits on diversification.

In the following paragraphs I do not pretend to make and extensive review of the speciation processes among different taxa and plants. Rather, I just exemplify these points of the evolution and speciation of insects around associations with plants: Lepidoptera and Coleoptera, with some exceptions in cases where the better examples come from other taxa (eg. Diptera, Hymenoptera and Hemiptera).

**At the ecological level**

**1) The nature of the interaction**

To describe the nature of the interaction I identify three components: the taxon under study, the ecological nature of the interaction, and the degree of specialization. The taxon under study have remarkable importance since each lineage has its own evolutionary basis, constrains and trends. The order or even the family of insects we are studying must be taken into account in the study of the insect-plant interaction. The ecological nature of the interaction refers to pollination (pollen collection), herbivory (external foliage feeding, feeding on internal tissues: piercing and sucking, boring, leaf mining, galling, seed predation), parasitism (oviposition site, stem borers), shelter or

protection (mimicry place) (Grimaldi, 2005; Herrera and Pellmyr, 2002). One would expect that each of these interactions to have different repercussions on the gene pool of the interacting species, depending on the degree of intimacy of the interaction, and the reciprocal influence exerted by each other exert (Stireman, 2005). In phytophagous insects the degree of specialization and generalization in host plant use must be placed within the context of the actual host range to that of available hosts (Vega & Hofstetter, 2015). It is assumed that specialization limits the breadth of resources an organism relies on and potentially results in increased isolation among populations, consequently having macroevolutionary consequences for lineage diversification (Forister, et al. 2012). Just as Erlich and Raven (1964) pointed out, changes in food plant choice would be specially favored in situations where the supply of the "preferred" plant is sufficiently limited to be an important factor in the survival of the larvae. The plant must be suspected to exert a strong selective pressure to the insect.

**At the microevolutionary level**.

**2) Characteristics that mediate the interaction and have an effect on local adaptation**

Several selective pressures promoting differentiation can act on different traits among species populations. It is expected that in traits that mediate the insect-plant interaction, the insect phenology must be related to host plant phenology (Abrahamson & Blair, in Tilmon, 2008). The identification of the traits that are mediating directional selection within the interaction, can give us an idea of the direction of evolutionary change. In fact, these traits depend on the nature of the interaction and they have to be directly related to fitness. In the literature there is not abundant evidence of the characteristics that mediate the interaction between insects and plants; the available mainly centers on Lepidoptera and Coleoptera.

Lepidoptera: They respond to the secondary metabolites of plants storing and detoxifying them. Species that store plant-derived pyrrolizidine alkaloids are found among moths *Arctiidae* and butterflies of subfamilies *Danainae* and *Ithomiinae* (Hartmann and Ober, 2000). Detoxifying enzymes has been found on *Manduca sexta,* the polysubstrate monooxigenases are non-specific detoxification enzymes rapidly induced by the presence of toxins, and the terminal component cytochrome P-450 which catalyzes the oxidation of toxins (nicotine in this case) to produce more polar compounds that are excreted or further metabolized (Snyder et al. 1994; Berenbaum, 1999).

Coleoptera: few subfamilies from Chrysomelidae release defensive compounds and in this family only some species of genus *Oreina* are known to sequester pyrrolizidine alkaloids (Hartmann and Ober, 2000). Whereas in Curculionidae there are no reports about how they deal with secondary compounds, but the rostrum length has been described as a key trait on insect-plant interaction and coevolution (Toju, 2011).

Diptera: only the corolla length and proboscis length have been described as traits that mediate the interaction, where reciprocal selection acts (Pauw, et al.2009).

**3) A form of reproductive isolation that links selection with genetic differentiation.**

The genetic mechanism that causes speciation and linking selection to reproductive isolation can be direct when the same genes under ecological selection cause reproductive isolation, or indirectly when the reproductive isolation is caused by effects of pleiotropy (including the speciation model of Bateson-Dobzhansky- Muller, or effects of poliploidization or hybridization).

The form of reproductive isolation on insects has been described mainly as non assortative mating, intrinsic hybrid unviability, and preferences in oviposition (Abrahamson & Blair, 2008; Matsubayashi, 2010). Cunningham (2012) proposes and explains the "skill" of the insect to choose the host plant through smell.

In Lepidoptera we have the *Heliconius* butterflies (Lepidoptera:Nymphalidae) where the mimetic wing color is under directional selection and individuals prefer to mate into those of the same color (Naisbit, 2001) which at the time is related to host plant preference and larval performance (Suppler et al. 2014).

In Coleoptera: in Chrysomelidae as well in Curculionidae it is supposed that the preference of insects to lay eggs on plants that are suitable for the development of their larvae (internal and external feeding habit of the larvae) is the isolating mechanism that causes reproductive isolation. But there is scare empirical evidence.

In Diptera: speciation in the genus *Rhagoletis* flies (Diptera: Tephiritidae) supports the idea that fidelity to the host plant can operate as a mechanism of pre and post copulatory isolation (Feder, 1994; Linn, 2004). Mate selection and host-plant are directly correlated (Bush, 1969; Linn, 2003).

**4) Intraspecific phylogeography**

The consideration of the genetic variation at the geographic distribution of the species involved allows to know the role of microevolutionary factors (e.g. migration or genetic drift) in the process of population differentiation. Phylogeographic studies facilitate the examination of the complex roles of geography associated to genetic breaks (Marske et al. 2013) as well as to identify genetic variation associated with plants (e. g. Hernandez 2010). The historicity and geography of the interaction allows us to better understand the relationship between insects and plants (Farrell 1990, Farrell et al. 1992; Thompson 2005).

At the same time that COI gene sequence is used in phylogeographic studies it also has been broadly used to look for genetic differentiation associated to host plants (Streiman et al. 2005; Hernandez 2010). It is quite important because in many cases there have been described "host forms" of herbivore individuals or populations, exhibiting host associated biological variation but the kind of variation has not been diagnosed (Funk, 2012), reports based on *Fst* do not allow to know the nature of the genetic variation.

In Lepidoptera: Crambidae grass moths show haplotypes associated to different host plant species but additional experiments are required to demonstrate species-specific interactions; until now, it just has been described that they show genetic differentiation associated with geographic regions along their distribution (Díaz-Montilla, et al. 2013). Others species as *Aglalis urticae* in spite they have variation in larval host plant species, they did not show geographic structure in a wide geographic distribution (Vandewoestijne, et al. 2004).

In Coleoptera: some Chrysomelidae insects do not show genetic variation associated to the host-plant, probably due to recent host-shift and multiple colonization events (Kohyama, et al. 2014; Kato, et al. 2010; Mardulyn, et al. 2011). Curculionidae otherwise show that plants can actually serve as a source of divergent selection related to host plant (Hernández-Vera et al 2010; Toju et al. 2011; Aoki et al. 2009). They also respond to microevolutionary factors (migration, genetic drift,

etc.) also (Aoki, et. al. 2010). Few studies on specialized Curculionidae do not show genetic variation associated to host-plant (Iwase et al. 2015).

An important point here is that even if genetic variation is associated with a clade, we do not known how long this conservatism will persist (Futuyma and Agrawal, 2009) and if the output will be speciation.

The model of the Geographic Mosaic of Coevolution proposed by Thompson (2005) has empirical evidence among insects and they associated plants (Toju et al. 2011). It modifies a little the output of selection: Landscapes may produce coevolutionary *hotspots* (regions where true reciprocal selection acts on both, insect and plant) embedded in a matrix of coevolutionary *coldspots*; in addition, the genetic landscape in which one interaction occurs may be constantly changing through gene flow, random genetic drift and local extinction of populations. As a result, few trails will spread across the whole geographic range of interacting species and this species can evolve in different ways in different populations (Thompson 1999). In Coleoptera, one of their main outputs in the interaction between *Curculio camelliae* and *Camellia japonica* it is that the co-evolving counterpart that is more vagile (weevil) tend to drive the coevolutionary dynamics of the interaction.

**At the macroevolutionary level.**

**6) Interspecific phylogeography**

By comparing several taxa of insects and plants distributed in the same area, it is possible to distinguish when the association started and if in fact, have a shared evolutionary history. It also permits the recognition of stochastic factors that influence the co-distributed species (Avise, 2004).

In Lepidoptera: There is not much evidence looking for phylogeographic association with plants in the same area of distribution. Probably this is due to their high vagility, as compared with others insects.

In Coleoptera: in Chrysomelidae there is no genetic structure associated to host plant since haplotypes do not show any pattern among the plants where they were collected, in fact there could be multiple colonization events (Mardulyn et al., 2011; Kohyama et al., 2014) because they are not

necessarily specialist. Several phylogeographical studies have been carried out in Japan, with Chrysomelidae and Curculionidae and their associated plants. It is very interesting that the nature of the interaction has effects on the phylogeographical patterns found among weevils (Sota et al., 2004; Kohyama et al., 2014; Aoki et al., 2011). In weevils distributed on islands the phylogeographic pattern seems to be more influenced by their local distribution and vagility but weevil habits are poorly known (Sequeira et al., 2012).

**7) Conservatism: coevolution, co-speciation and colonization**

When did the interaction begin and for how long it remained? Molecular dating shows two patterns colonization and cospeciation. When the clade of insects is younger that the plants to which they are associated this interaction is due to colonization where as the fit among topologies and divergence times between insects and plants are interactions explained by cospeciation (de Vienne et al., 2012). Coevolution in strict sense, should be corroborated testing reciprocal selection pressures. Phylogenetic studies of highly specific insect-plant association are scarce for Lepidoptera and even Coleoptera; the most well-known examples comes from Hymenoptera and Hemiptera:

Hymenoptera: The well-known example of phylogenetic correspondence is found and described among *Ficus* plants and their pollination wasps (*Ceratosolen*). Reconciled phylogenies show 13 cospeciation events, one host switch and four duplications. The same *Ficus* show less congruence with the phylogeny of their nonpollinating gallers, stressing the importance of the ecological nature of the interaction (Silvieus et al., 2008).

Hemiptera: in psyllids, colonization and cospeciation seems to be more common than coevolution among their host plant (legumes). Among four main legume lineages that speciated before psyllids, most of them were colonized by the insects and only a single event of cospeciation is probable (Percy et al., 2004). The ecological factors that may have influenced the host switching are: host population size, geographical proximity and unoccupied host.

In Lepidoptera: One of the main examples of cospeciation in butterflies it is not related with the host plant, but similar selection pressures among species is the case of the Müllerian mimicry in

*Heliconius* butterflies, a study made at population level between *H. melpomene* and *H. erato* (Cuthill and Charleston, 2015).

In Coleoptera: among Chrysomelidae there are few comparative phylogenetic studies reported, so far, the phylogenies of *Blepharida* and it host plant, *Bursera*, suggest that diversification of insect lineages follow the complex chemistry of their host plants in a recent adaptation event, contrary to the expected coevolution among plant species (Becerra, 2003). Curculionidae and plants have been of great interest due to its extraordinary diversity and the phylogenetic studies. Some of the major findings are the existence of a temporal lag among diversification of angiosperms and weevils (MacKenna et al. 2009). Associations that were supposed to be very old are recent radiations (Downie et al., 2008). Also, it is suggested to focus on generic or tribal phylogenies in order to obtain reliable inferences about its evolutionary success on plants (Franz and Engel, 2010).

**8) Mapping key traits on diversification**

On insect phylogenies as well as in plant phylogenies it is possible to map the traits that are believed to have a role in diversification at a higher taxonomic level, and in this way to make hypotheses on important characters mediating the interaction (Armbruster, 1992). Also, phylogenies can serve to perform independent contrasts, and identifying traits whose occurrence is correlated with evolutionary changes in host range. At the same time, the rate and direction of change can be estimated (Armbruster, 1992; Winkler & Mitter, 2008).

Studies assessing ancestral reconstruction of host plant and traits associated to the interaction are common in Lepidoptera (Nylin et al., 2013) and Coleoptera (Kato et al., 2010; Kobayashi et al., 2012;).

**The ideal scenario of insect-plant speciation.-Conclusion**

At some point, two lineages (insect and plant) start to influence each other's fitness. The selection pressure promotes population differentiation that in some cases end with speciation. There may be several selection agents acting on these lineages and they can vary at different developmental stages, probably not in the same intensity, in phytophagous insects it is expected that host plant be the one

acting with more intensity, but like in other organisms phytophagous insects are under the effects of genetic drift too.

It is difficult to have a clear picture of the speciation process in insects, taking into account these diverse selective factors but there are some good studied systems as *Heliconius* butterflies which has attempted to explain speciation process considering: selective factors (host-plant preference and physiological traits in larval, matting preference and cryptic morphology of adults), gene flow and hybridization, and their impact on genomic heterogeneity of divergence (Supple, et. al. 2014).

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**Speciation in weevils**

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Weevils constitute the second largest family group of the animal kingdom. They are among the most successful and multitudinous forms of terrestrial life. Have been described around 62 000 species and has been estimated there are 220 000 extant species (Oberprieler et al., 2007). This diversity has been associated to the diversification of flowering plants, mainly angiosperms (Farrell 1998). Curculionidae originated about 171.5+/- 27.07 m. a. at the end of the Jurassic, but the main diversification occurred in the Cretaceous (140-60 m.a.) invading a wide range of niches, including the utilization of all parts of plants (Hunt et. al., 2007; McKenna et al., 2009). Weevils feed from roots, bark, sapwood, heartwood, stems, twigs, buds, flowers, pollen, fruits, seeds and sick, dying, dead and decaying plant material (Oberprieler et al., 2007).

Traditionally seven major weevil lineages have been recognized and supported by molecular data (Malvardi et al., 2002): Nemonynchidae, Anthribidae, Belidae, Attelabidae, Caridae, Brentidae and Curculionidae. The Nemonynchidae family is the oldest and presumably retain the ancestral life style of weevils, the eggs are laid openly on the cones (of conifers) and their mobile larvae live freely among the sporophylls, feeding on pollen in the open sporangia and moving between cones. Adults also feed mainly on pollen. The Anthribidae family is characterized by mycetophagy, adults and larvae develop predominantly on wood-decaying ascomicete fungi, the eggs being inserted into the plant tissues by means of specialized dentate ovipositor at difference of others weevils where the rostrum is used to prepare oviposition site. The rostrum of these weevils is seemingly adapted to grazing fungal mycelia. The Belidae family and the remaining families have the rostrum transformed into a proper oviposition tool by the fusion of the labrum and the clypeus and the development of more advanced mandibles with long pharingeal processes, enabling their eggs to be deposited inside firm plant tissues, and their larvae develop truly endophytically. The larvae of Belidae are woodborers in stems and logs, the more primitive mostly in conifers and the others in angiosperms (araucarias and cycads). The Attelanidae family is also associated with fungi, their larvae develop on withering plant tissues that may be indirectly or directly infested with fungi. In

contrast to athribids, female attelabids prepare their ovioisition site with the rostrum; in advanced ones females roll up leaves into elaborated, cigar-like nidi (nest) in which they lay their eggs and can inoculate with fungal spores carried in a special mycetangia next to their hind coxe. These weevils are predominantly associated with angiosperms; only primitive ones are found living in conifers. The Caridae family is considered a relict group; host associations are predominantly with Cupressaceae conifers. The larva develops in young seeds in closed female cones where females drill oviposition holes with their rostrum. The Brentidae family is almost exclusively associated with angiosperms. The monophyly of this group remains in dispute, the larva develops in a variety of environments some of them feed on roots in the solid, in living or dead branches and others in young stems and inflorescences, fruit or seed pods. Finally, the Curculionidae family feed on virtually all plants, mainly angiosperms; their larvae predominantly live an endophytic life inside all parts of plants and some adopted a more ectophytic life; the larva feeding exposed on leaves or in the soil on roots and a few have a specialized life style such as coprophagy, myrmecophily and even predation. In this group most of weevils use their rostrum to prepare or better said, to drill the oviposition site. They also possess a geniculate antenna with a compact antennal club (family feature) which plays an important role in the exploitation of host plant tissues. A geniculate antenna allows to drill deeper, whereas the club selects the exact location on the host plant where the tip of the rostrum is to begin drilling the oviposition hole (Oberprieler et al., 2007).

Based on this information Oberprieler et al., (2007) proposes a hypothesis of weevil evolution based on key factors in addition to the angiosperms diversification (Figure 1). They hold that there is no single and simple explanation for the huge diversity of weevils but rather a cascade of evolutionary innovations responsible for their success. Briefly, in the Cretaceous the evolution of the rostrum and the rise of angiosperms were crucial and major evolutionary events, in the Jurassic the diversification of conifers and their protective strobili advantage weevil diversification and probably the association with fungi too. In the Tertiary explosive radiation of eucotyledons was possible due to evolution of **oviposition rostrum** and **endophytic larva**, avoided desiccation problems on immature stages but also promoted a response to a variety of plant defenses (Anderson, 1993). Recently, the evolution of the **geniculate antenna** in high weevils allowed the possibility to exploit the angiosperm diversity. The rostrum is used to drill the holes to oviposition, the physiology of the larvae has to deal with chemicals defenses of plants where it develops (e.g. secondary metabolites),

and the antenna is a perception organ that can be adapted to perceive secondary metabolites together with the functional ability to blend in order to allow deeper holes to oviposition.

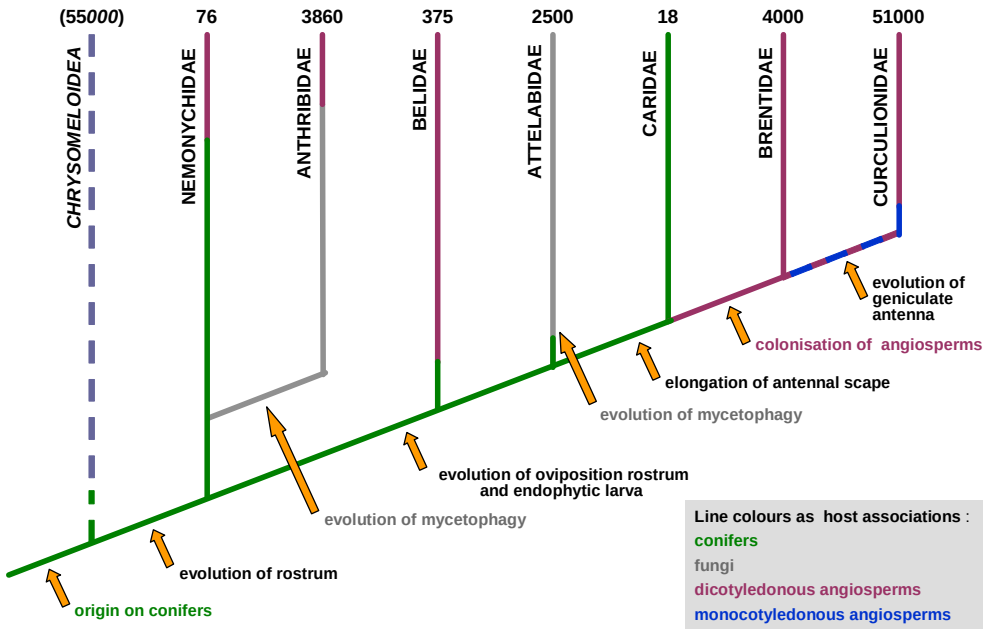


Figure 1. Key evolutionary events in the diversification of the Curculionoidea mapped onto their phylogeny, with numbers of described species for each family (taken from Oberprieler *et al.*, 2007).

Thus, the study of host associations between weevils and plants gives support to the study of weevil speciation, and assessing for example the importance of cospeciation versus colonization. The weevils represent a group well suited for these type of studies where the host plant can be an evolutionary selection pressure. In Curculionidae species diversity is high, many, if not most, angiosperm taxa serve as a hosts, many lineages exhibit a high degree of association with a limited range of plant hosts, many species are associated with a limited number of hosts and some may prove very narrowly oligophagous or monophagous and thus cospeciation is at least possible (Anderson, 1993). However, the study of weevil evolution in this context is difficult because there are many factors involved in host plant associations. For example, the host plant is known with some degree of certainty for most species in relatively very few groups of weevils and variation in host association in the same species of weevil is inadequately known (Anderson, 1993).

If it is assumed that a particular genus of weevils is associated with a restricted taxonomic range of host, then at least some, if not all, of the evolution of these host associations can be explained through cospeciation. On the contrary, others factors must have mediated weevil evolution, such factors could be geographical range or habitat (Anderson, 1993). Geographical factors that mediate weevil evolution together with host associations can explain the genetic divergence between weevil populations that sometimes can result in speciation.

Some studies aimed to identify genetic structure associated to host plant. Taking into account the characteristics (kind and specificity) of the interaction, it is possible to distinguish what geographical factors influence the genetic variation and, at the same time, which plant-herbivore relationships has been largely maintained, where the genetic variation has been constrained the due to local natural selection.

Host-specific seed predators like *Curculio hilgendorfi* and *C. camelliae* show genetic differentiation associated to their host plant, their genealogical lineages have been shaped by environmental factors (Aoki, et al. 2011; Toju and Sota, 2006). The host-specific inner bark, *Dentroctonus pseudotsugae*, presents a phylogeographic pattern that can be explained by past fragmentation of their host plant (Ruiz, et al. 2010). Others ways of interaction like in the host-specific leafminer, *Rhynchaenus dorsoplanatus*, show a genetic structure that is not see associated to its host plant (Aoki, et al. 2011), neither to the environmental history of its distribution area, probably due to leaves drift (Aoki, et. al. 2010).

In contrast host-generalist like *Curculio sikkimensis, Dendroctonus mexicanus* and *Dendroctonus approximatus* show differentiation patterns that seems to be responding to the geological history and climatic conditions of their current distribution (Aoki, et al. 2011; Anducho-Reyes et al. 2008; Sánchez-Sanchez et al. 2012).

Key traits for evolution of weevils such as the rostrum, larva physiology and antennae, have been explored in order to establish coevolutionary relationships. The rostrum length has been involved in a geographically-structured arms race (Toju and Sota, 2006), since this trait direct has affects at local level the fitness of both weevils and host plant (Toju, 2009). In *Curculio camelliae* the rostrum length interacts with the pericarp width of its host plant (drills to oviposit the eggs), the differentiation of the rostrum length is due to historical events and local selection exerted by host defense. This study system has been broadly explored (Toju and Sota, 2006; Toju and Sota, 2009;

Toju, 2009; Iseki, et al. 2011; Toju, et al. 2011a; Toju, et al. 2011b; Toju, 2011). The macroevolutionary level reveals that variation in rostrum length has been evolutionary stable in length but when there is a selective pressure on it, this will accelerate its growth (Toju and Sota, 2009). At the microevolutionary level and in a smaller area of its current distribution and according to the geographic mosaic theory of coevolution (Thompson, 1994), there are some populations where selective pressure among populations is stronger than in others, and the counterpart more vagile is who drive the coevolutionary dynamics (Toju, et al. 2011b). It is hard to identify such traits, for example in *Stona gressorius* did not show discrimination between alkaloids of different lupine species where they feed (Ströcker, et al. 2013). Probably because the evolution was made in adult stage and because the larvae develops on roots where it is expose to others selective factors.

The formation of insect races has been found as evidence that this interaction insect-plant could promote speciation, for example in the weevil *Euhrychiopsis sibiricum* the host races are formed more or less quickly, 33 generations, associated to their host plant. This in fact shows that natural selection can drive adaptive divergence on an ecological time-scale. In this case host plant can manifest reproductive barriers in dozens to hundreds of generations (Hendry et al. 2007).
The knowledge of the speciation process in weevils is very important to biodiversity conservation and pest management. And as we can appreciate the host-plant is an important source of selection but not the only one, there is of need a more integrative approach to understand the speciation process in this extraordinary family of beetles.

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## SISTEMA DE ESTUDIO

El género *Trichobaris* LeConte es un grupo de coleópteros pertenecientes a la familia Curculionidae, subfamilia Baridinae, tribu Baridini, esta última es especialmente diversa en los trópicos americanos (Oberprieler et al., 2007). Las especies de este género se distribuyen en Estados Unidos y México, es decir en la región Neártica del continente americano, teniendo como límite sur el Itsmo de Tehuantepec (Barber, 1935).

Se han identificado entre 9 y 28 especies (EOL; O'Brien and Wibmer, 1982; Barber, 1935; GBIF; Zipcodezoo), aunque Barber (1935) es el único que ofrece una breve descripcion de cada una junto con un mapa de su distribución. Al mismo tiempo propone una clave dicotómica para la identificación de las 13 especies. Este grupo de curculiónidos se alimenta de los tejidos de varias especies de plantas de la familia Solanaceae, en particular, las especies del género *Datura* L. De las 13 especies de *Trichobaris*: 6 parasitan frutos de las especies del género *Datura,* 3 tallos de cultivos de papa, tabaco y tomate, y 4 tallos de plantas silvestres (*Solanum elaeagnifolium, S. rostratum* y *S. carolinense*) (Barber, 1935).

El ciclo de vida de estos escarabajos consiste en que la hembra perfora el tejido de la planta hospedera (dependiendo de la especie el tallo o el fruto), y deposita ahí un huevo por perforación. Las larvas en desarrollo se alimentan del tallo o las semillas, según sea el caso, hasta alcanzar el estado de pupa. Dentro de los tejidos de la planta se mantiene resguardada la pupa, en una cápsula hecha con heces de la larva, hasta su metamorfosis como adulto, cuando sale de la planta hospedera a reproducirse. El adulto se alimenta de las hojas y flores de la misma planta (Vargas-Cabrales, 1991; Diezel, et al. 2011; Cuda and Burke,1986; 1991).

Entre las especies de *Trichobaris* existe variación en cuanto a la amplitud de las especies de plantas en las que depositan sus huevos, algunas son especialistas en la especie de planta donde se desarrollan, mientras que otras se desarrollan en varias especies de plantas (Barber, 1935; Cuda and Burke,1986; J. Núñez-Farfán, obs. pers.). No obstante, la planta huésped no ha sido corroborada en todos los casos.

## PLANTEAMIENTO DEL PROBLEMA

De acuerdo a la hipotesis general de que la diversificación de los curculiónidos es debida en gran medida a la interacción con las angiospermas (MacKenna, 2009) la estructuración genética entre las especies de *Trichobaris* podría diferir entre un especialista y un generalista. Se ha propuesto que las especies especialistas tendrán menos variación genética en general, y se encontrarán asociaciones a la planta huésped en su estructura genética, contrario a lo que se espera para especies generalistas dónde se espera mayor variación genética y la ausencia de asociaciones en los distintos huésped (Kelley et al. 2000). La repercución macroevolutiva sugiere que en los insectos herbívoros oligo o monófagos las asociaciones con sus plantas huésped pueden contribuir a la formación de razas de huésped genéticamente distintas y en última estancia provocar especiación (Erich & Raven, 1964).

## HIPÓTESIS

La planta huésped influyó en la variación genética del género *Trichobaris* en dos niveles evolutivos:
A nivel microevolutivo las especies especialistas tendrán menos variación genética y ésta se encontrará asociada con la planta huésped. Lo contrario se espera de especies generalistas donde habrá mayor variación genética y ausencia de asociaciones genéticas en las diferentes especies de plantas huéspedes.
A nivel macroevolutivo las especies de *Trichobaris* especialistas mostrarán clados asociados con plantas huéspedes recientes mientras que las generalistas mostrarán paralelismos asociados con plantas huéspedes recientes.

## OBJETIVOS

Identificar los patrones y procesos que gobiernan la distribución geográfica de los linajes genealógicos en dos especies de *Trichobaris* que varían en su especificidad con la planta huésped.

Estimar las relaciones evolutivas entre las especies de *Trichobaris* y con su planta huésped.

# Artículo de investigación 1

Genetica
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# Phylogeography of specialist weevil *Trichobaris soror*: a seed predator of *Datura stramonium*

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**Abstract** Can the genetic structure of a specialist weevil be explained by the geological history of their distribution zone? We analyze the genetic variation of the weevil *Trichobaris soror*, a specialist seed predator of *Datura stramonium*, in order to address this question. For the phylogeographic analysis we used the COI gene, and assessed species identity in weevil populations through geometric morphometric approach. In total, we found 53 haplotypes in 413 samples, whose genetic variation supports the formation of three groups: (1) the Transmexican Volcanic Belt (TVB group), (2) the Sierra Madre Sur (SMS group) and (3) the Balsas Basin (BB group). The morphometric analysis suggests that BB group is probably not *T. soror*. Our results have two implications: first, the phylogeographic pattern of *T. soror* is explained by both the formation of the geological provinces where it is currently distributed and the coevolution with its host plant, because the TVB and SMS groups could be separated due to the discontinuity of altitude between the geological provinces, but the recent population expansion of TVB group and the high frequency of only one haplotype can be due to specialization to the host plant. Second, we report a new record of a different species of weevil in BB group parasitizing *D. stramonium* fruits.

**Keywords** Coevolution · *Datura* · Phylogeography · *Trichobaris* · Weevil

## Introduction

The genus *Trichobaris* (Curculionidae: Subfamily Baridinae; LeConte 1876) has 13 species, all of which are distributed in the Neartic region; no species of *Trichobaris* are known south of the Isthmus of Tehuantepec in Mexico (Barber 1935). Some species of *Trichobaris* feed upon cultivated solanaceous plants: tobacco (host plant of *T. mucorea*), potato (host plant of *T. trinotata*), and tomato (host plant of *T. championi*). Infestation of cultivated plants by *Trichobaris* spp. is thought to be a result of the local transference of weevils from their wild solanaceous host plants (Barber 1935). In contrast some other species of *Trichobaris* are restricted to a particular host plant species where they feed, breed, and develop (Cuda and Burke 1991; Cabrales-Vargas 1991). An example of this is *Trichobaris soror*, the life cycle of this species is tightly associated to jimsonweed, *Datura stramonium*. The adult feeds on leaves and stems of the plants whereas the larva feeds on developing seeds inside the fruits (Cabrales-Vargas 1991). Although *Datura stramonium* is known as distributed worldwide (Warwick 1990) their original distribution encompassed temperate regions of the Mexican Plateau and few warm places ( Barclay 1959b). *Datura* diversified in Mexico (Barclay 1959a). Despite of the broad distribution of its host plant *Trichobaris soror* is restricted to the Mexican highlands (Barber 1935) where its host plant occurs. Such distribution makes the analysis of the phylogeography of this weevil species particularly interesting. The recent geological history of Mexico could have had an impact on its genetic structure and historical demography.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10709-015-9866-x) contains supplementary material, which is available to authorized users.

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The main physiographic features of Mexico attained their present geomorphological configuration between Early and Late Miocene (23–5 mya). These include the Sierra Madre Occidental, Sierra Madre Oriental, Transmexican Volcanic Belt, Sierra Madre del Sur and Highlands of Chiapas provinces (Padilla et al. 2007; Cevallos-Ferriz and Gonzáles-Torres 2005). During the Pliocene, the completion of the Panamanian Isthmus, bridging North America and South America was established (3.5 mya; Collins et al. 1996), giving rise to biota interchanges between the Neartic and Neotropic biotas (Morrone, 2015). This recent geological history could have affected biodiversity of coleopterans in the zones where the Neartic and Neotropical regions overlap, Mexican area (Halffter 1964).

However, to date few phylogeographic studies of coleopterans in Mexico have been carried out. The phylogeographic pattern of *Saltator limbatus*, a generalist seed predator of legumes (Fabaceae), can be explained by the geological history of its distribution (Morse and Farrell 2005). Most of the species of *Dendroctonus* are also generalists (Kelley and Farrell 1998), and feed on living and dead phloem tissues of *Pinus* spp. They reveal different routes of colonization from North America and genetic clusters associated to Mexican geological provinces (Mock et al. 2007; Sánchez-Sánchez et al. 2012). In addition, *D. ponderosae* and *D. aproximatus* also show a genetic clustering associated to host plants (Mock et al. 2007; Sánchez-Sánchez et al. 2012).

*Trichobaris soror* is a particularly interesting species for phylogeographic studies because, in addition to the geological processes that may have influenced its genetic variation, there is a specific host relationship with *D. stramonium.* It has been postulated that the intensity of a co-evolutionary relationship can vary among populations and change the frequency of haplotypes in these populations (see coevolution, in Thompson 2005). Therefore, ideally the phylogeographic pattern of *T. soror* and *D. stramonium* should match each other (v.gr., Toju et al. 2011), and fit with the geological history of their distributions.

In this study, we aimed to assess the phylogeographic and genetic structure of *T. soror*, we do this by analyzing the variability of a segment (658 bp) of cytochrome c oxidase subunit I (*COI*), a mitochondrial gene. We also estimated the divergence times of distinct lineages using a fixed rate of mutation rate reported for the *COI* gene. Finally, we perform a geometric morphometric analysis of male genitalia to assess if other species *Trichobaris* parasite *Datura stramonium.*

## Materials and methods

### Study site and insect collection

*Trichobaris soror* is distributed throughout the highlands of central Mexico. Because *T. soror* is a seed predator of *D. stramonium*, we sampled 14 populations of the plant from August to December 2010 (Table 1). In each population of *D. stramonium*, all fruits from each of 30 plants were collected, individually bagged and labeled. Females lay from one to several eggs per fruit so to perform genetic analysis we took only one weevil from each fruit, in order to avoid sampling relatives. Nine insects of *Trichobaris compacta* were sampled on *Datura reburra* plants to be used as an outgroup (Table 1). Twenty specimens of *T. soror* were donated to the entomological collection of the Biology Institute of the National Autonomous University of Mexico (UNAM).

### DNA extraction, PCR and sequencing

Each sample was frozen with liquid nitrogen (−196 °C) and then macerated with a micropestle. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen™) according to the manufacturer's protocol for animal tissue.

We then amplified a region of 658 nucleotide protein coding region of the mitochondrial gene cytochrome c oxidase I (*COI*) by polymerase chain reaction (PCR) using the following primers: COA3107 (TCT ATT ARD GGD GAD GCD CTA TCT TG) and COS2183N (CAR CAY YTA TTY TGR TTY TGR TTY TTY GG) (Sota et al. 2004).

The polymerase chain reactions (15.6 µl in volume) each contained 1 µl DNA (20 nM), 1 µl each primer (10 mM), 0.5 µl Taq DNA polymerase (Invitrogen™), 0.5 µl each nucleotide (10 mM), 1.5 mM $MgCl_2$ (3 mM) and 8.6 µl $H_2O$. The thermal cycling conditions were as follows: an initial period of 5 min at 95° C, followed by 35 cycles of 60 s at 95 °C, 1.2 min at 55 °C, and 60 s at 72 °C, with a final extension for 7 min at 72 °C. PCR products were sequenced at Washington University using an ABI 3730xl sequencer (Applied Biosystems™). All nucleotide sequences obtained were compared, edited manually with Sequencher™ 4.7 software, and aligned with CLUSTAL W (Thompson et al. 1997).

### Genetic diversity

To compare the levels of diversity of *T. soror* populations (Table 2), we estimated the total number of haplotypes,

**Table 1** Sampled populations of *Datura stramonium* (host plant), number of insects sampled and genetic variability of *Trichobaris soror*, based on mtDNA (*COI* gene) sequences

| Number | Locality | State | North | West | Altitude | Number of *D. stramonium* | Number of *T. soror* | *S* | Singletones | # mutations | # haplotypes | *h* | $\pi$ | $\theta$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Tlaxiaca (Tla) | Hidalgo | 20 08 00.46 | −98 52 18.56 | 2353 | 50 | 39 | 7 | 5 | 7 | 4 | 0.317 | 0.00089 | 0.00252 |
| 2 | Francisco Villa (FV) | Hidalgo | 20 42 09.64 | −99 18 12.28 | 1962 | 44 | 24 | 3 | 1 | 3 | 3 | 0.409 | 0.00132 | 0.00122 |
| 3 | Dios Padre (D) | Hidalgo | 20 27 55.05 | −99 09 21.39 | 1726 | 16 | 16 | 23 | 21 | 23 | 4 | 0.692 | 0.00541 | 0.00105 |
| 4 | Patria Nueva (PN) | Hidalgo | 20 22 35.26 | −99 02 40.24 | 1911 | 41 | 33 | 5 | 3 | 5 | 5 | 0.449 | 0.00147 | 0.00187 |
| 5 | El Tepe (PT) | Hidalgo | 20 27 03.66 | −99 10 19.70 | 1941 | 19 | 17 | 4 | 4 | 4 | 4 | 0.331 | 0.00072 | 0.0018 |
| 6 | Valsequillo (Val) | Puebla | 18 58 30.08 | −98 10 09.28 | 2163 | 50 | 50 | 10 | 2 | 11 | 8 | 0.633 | 0.00372 | 0.00339 |
| 7 | Atlixco (Atl) | Puebla | 18 54 37.85 | −98 26 04.77 | 1858 | 45 | 33 | 25 | 2 | 28 | 8 | 0.513 | 0.00576 | 0.00936 |
| 8 | Panzacola (H) | Puebla | 19 09 11.57 | −98 13 06.11 | 2188 | 42 | 34 | 13 | 6 | 13 | 12 | 0.781 | 0.00423 | 0.00483 |
| 9 | Vía (Vía) | Puebla | 19 21 19.24 | −98 06 34.96 | 2315 | 36 | 37 | 13 | 6 | 13 | 12 | 0.781 | 0.00423 | 0.00483 |
| 10 | Xochipala (Xo) | Guerrero | 17 47 09.76 | −99 39 42.01 | 1108 | 45 | 20 | 2 | 0 | 2 | 2 | 0.268 | 0.00082 | 0.00086 |
| 11 | Rancho Colores (RC) | Guerrero | 17 44 07.98 | −99 29 47.68 | 1443 | 44 | 31 | 30 | 5 | 31 | 10 | 0.794 | 0.00981 | 0.01141 |
| 12 | Teotihuacan (Teo) | Edo. de México | 19 41 32.25 | −99 50 37.27 | 2551 | 38 | 29 | 11 | 7 | 11 | 8 | 0.48 | 0.00172 | 0.00426 |
| 13 | Morelia (Mo) | Michoacán | 19 36 13.25 | −10117 5.24 | 1905 | 30 | 25 | 25 | 22 | 25 | 7 | 0.627 | 0.00415 | 0.01006 |
| 14 | Oaxaca (Oax) | Oaxaca | 16 50 28.97 | −96 16 57.77 | 1997 | 25 | 25 | 18 | 1 | 18 | 5 | 0.47 | 0.00872 | 0.00724 |
| | | | | | | Total | 413 | 56 | 18 | 62 | 53 | 0.722 | 0.01013 | 0.0129 |
| 15 | Guamuchi | Sinaloa | 25 27 40.28 | −108 4 45.97 | 48 | *D. reburra* | T. compacta n = 9 | 7 | 5 | 7 | 5 | 0.833 | 0.00329 | 0.00391 |

The outgroup (one sampling site) is *Trichobaris compacta*

*S* segregating sites, *h* haplotype diversity, $\pi$ nucleotide diversity, $\theta$ diversity index

**Table 2** Wright's $F_{ST}$ pairwise values among populations of *Trichobaris soror*

| $F_{ST}$ | Atl | Val | Teo | H | Via | D | PN | Tla | FV | PT | Mo | Oax | Xo | RC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Atl | 0 | | | | | | | | | | | | | |
| Val | 0.0352 | 0 | | | | | | | | | | | | |
| Teo | 0.0402 | 0.153* | 0 | | | | | | | | | | | |
| H | 0.0263 | −0.0108 | 0.1598* | 0 | | | | | | | | | | |
| Via | 0.0175 | −0.0148 | 0.1346* | −0.0182 | 0 | | | | | | | | | |
| D | 0.0153 | 0.0942 | 0.1380* | 0.076* | 0.0763 | 0 | | | | | | | | |
| PN | 0.0664* | 0.1534* | 0.1098* | 0.1542* | 0.1382* | 0.0268 | 0 | | | | | | | |
| Tla | 0.0434* | 0.1528* | −0.0036 | 0.1638* | 0.1370* | 0.15162* | 0.1108* | 0 | | | | | | |
| FV | 0.0554* | 0.1445* | 0.1078* | 0.1440* | 0.1283* | 0.0232 | −0.0315 | 0.1102* | 0 | | | | | |
| PT | 0.0223 | 0.1346* | −0.004 | 0.1392* | 0.1152* | 0.0897* | 0.0584 | 0.0075 | 0.0619 | 0 | | | | |
| Mo | 0.2573* | 0.3375* | 0.4713* | 0.3066* | 0.3234* | 0.1519* | 0.3663* | 0.5018* | 0.3682* | 0.4166* | 0 | | | |
| Oax | 0.4413* | 0.4624* | 0.6303* | 0.4219* | 0.4472* | 0.4353* | 0.6219* | 0.6581* | 0.5972* | 0.5877* | 0.4903* | 0 | | |
| Xo | 0.8780* | 0.9121* | 0.9703* | 0.9096* | 0.9121* | 0.9091* | 0.9638* | 0.9738* | 0.9679* | 0.9773* | 0.9125* | 0.7739* | 0 | |
| RC | 0.7273* | 0.7849* | 0.8153* | 0.7600* | 0.7695* | 0.7128* | 0.8170* | 0.8338* | 0.8011* | 0.7889* | 0.7371* | 0.5458* | 0.0934* | 0 |

Population acronyms follow Table 1

* $P < 0.001$

mutations, segregating sites ($S$), nucleotide diversity ($\pi$), haplotype diversity ($H$) and theta ($\theta$) using the Nei's (1987) equations implemented in version 5.1 of the DNAsp program (Rozas 2003).

## Genetic structure

After estimating the differentiation index ($F_{ST}$) between populations, we used Arlequin ver. 3.11 (Excoffier et al. 2005) to apply a Mantel test to analyze the correlation significance between genetic distance ($F_{ST}/1 - F_{ST}$) and the geographic distance among populations. We conducted a Bayesian clustering analysis in Structure 2.3.4 (Pritchard et al. 2000) and used the ΔK value to determine the number of genetic groups (Evanno et al. 2005). The COI haplotypes were recorded as a single locus, and the individuals were probabilistically assigned to one of the predefined $K$ populations (gene pools), to identify the optimal number of genetic groups. The optimal number of groups ($K$) was determined by varying the value of $K$ from 1 to 10 and running the analysis ten times per $K$ value to determine the maximum value of posterior likelihood [lnP(D)]. Each run was performed using $10^4$ burn-in periods and $10^6$ MCMC replicates after burn-in. We used the admixture ancestry model with correlated allele frequencies to determine the most probable $K$ value.

To improve the estimation of genetic groups we used a SAMOVA approach (Spatial Analysis of Molecular Variance; Dupanloup and Excoffier 2002), which defines populations that are geographically homogeneous and maximally differentiated from each other. The method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences among groups of populations. Simulations have shown that the SAMOVA algorithm does indeed find maximally differentiated groups, especially when data derive from a single locus.

## COI haplotype network and phylogenetic analysis

To visualize the frequency and distribution of haplotypes we constructed an un-rooted haplotype network (Bandelt et al. 1999), using Median-joining algorithm; the analysis was performed in Network v. 4.6.1.1. The ancestral haplotypes were inferred in TCS v.1.13 software (Clement et al. 2000) using a connection limit of 95 % statistically parsimonious (Figure S10; Supplementary Material).

Using only the haplotypes and not their frequency we performed a phylogenetic analysis. To compare topologies and the clade support of trees inferred we used Neighbour-Joining, Maximum Parsimony (PAUP software, v.4.0b2; David Swofford, Smithsonian Institution) and Bayesian Inference. The best-fit model of DNA substitution for

Bayesian Analysis was selected based on the Akaike information criteria (AIC) (jModel Test; Posada 2008). The MCMC algorithm was run for 1,000,000 generations with four incrementally heated chains, starting from random trees and sampling one out of every 100 generations. Posterior clade probabilities were used to assess nodal support (MrBayes 3.1.1. software; Huelsenbeck and Ronquist 2001).

In order to identify ancestral and derived haplotypes in the phylogeny, the trees were rooted using *Trichobaris compacta*. We estimated the divergence time between the main haplotype clades using a fixed substitution rate of 2 % per million years for COI, as was previously reported for coleopterans (Nakamine and Takeda 2008), and using a GTR + γ + I model of nucleotide substitution. The parameters of MCMC runs were 30 million generations, sampling every 1000 generations and discarding the first 10,000 trees as burn-in. The samples were summarized in the maximum clade credibility tree (BEAST v.1.4.7 package software; Drummond and Rambaut 2007). We visualized the results using Figtree 1.0 (Rambaut 2006).

### Historical demography

Assuming neutrality, we estimated the Fu and Li's *F* (1993) and Tajima's *D* statistics in order to infer population demographic history. The Tajima's *D* statistic is expected to be negative when genetic structure has been influenced by rapid range expansion, positive when the population has passed through a "bottle-neck", and zero when there is equilibrium between mutation and drift (Tajima 1989). To compare observed frequencies of pairwise differences with those expected under a model of demographic expansion, mismatch distributions were generated using DnaSP v.4.10 (Rozas 2003). A multimodal distribution is expected when there are no changes affecting population size, but unimodal distributions are expected when sudden demographic expansions have occurred (Rogers and Harpending 1992).

### Geometric morphometric analysis

Male genitalia, called *aedeagus*, was extracted from 35 individuals collected within the putative distribution of *T. soror* (Barber, 1935) but not all populations in the phylogeographic analysis were included in the morphometrics analysis because the aim of this analysis was to test if the insects from Balsas Basin were *T. soror*. Populations analyzed were: Atlixco, Panzacola, Via, Patria Nueva, Tlaxiaca and Rancho de Colores (from BB group, see Results section).

We took two pictures, one lateral and other frontal of the *aedeagus* of each specimen with AxioVision LE software [(c) Copyright Carl Zeiss Imaging Solutions GmbH, 2006], using a stereoscopic microscope and Cannon PowerShot A620 camera.

In order to reduce variance in landmarks positioning all were assigned by the same person and repeated with a second picture. The morphometric analysis was performed using both pictures. In total there were nine landmarks for the lateral view in each specimen, these landmarks were collocated using TPSdig v.1.40 software (© 2000 F. James Rohlf) (see Supplementary Material for the positioning of landmarks).

The differences in size were determined by the centroid size. To test differences in shape we calculated the Procrusters fit and constructed a covariance matrix. To test differences among populations we used an ANOVA from a principal component analysis (PCA) and a canonical variate analysis to cluster the shapes according to the population of origin. All of these analyses were performed using the MorphoJ software (Klingenberg 2011).

## Results

### COI variation and population structure

We sequenced 658 bp of COI mtDNA in 413 individuals of *Trichobaris soror* from 14 localities. We found 53 haplotypes, 56 segregation sites (*S*), 18 singletons, 38 parsimony-informative sites, and 62 inferred substitutions, excluding the outgroup (Table 1). We did not find indels or gaps. *T. soror* populations have high genetic diversity ($h = 0.722$, $\pi = 0.01013$ and $\theta = 0.01428$). Only four haplotypes are shared between populations (h1, h2, h7 and h9; Fig. 1), where the most abundant haplotype (h2) is present in 50 % of total sample. All populations had more than one haplotype (Table 1; Fig. 1).

High and significant genetic differentiation among populations was detected ($F_{ST} = 0.731$), however RC and Xo populations are highly influential on this estimate (Table 2). Genetic differentiation ($F_{ST}/1 - F_{ST}$) was not significantly correlated with geographical distance (Mantel's correlation coefficient $= -0.2605$ ($p = 0.001$); Fig. S1, Supplementary Material).

Bayesian clustering analysis detected three groups: I. Transmexican Volcanic Belt group (TVB group) conformed by Tla, D, FV, PN, PT, Val, Via, Teo, Atl, H and Mo populations; II. Sierra Madre del Sur group (SMS group) conformed by Oax population; and III. Balsas Basin group (BB group) conformed by Xo and RC populations (see Table 1 for population's abbreviation) (Fig. S2 Supplementary Material). The BB group (III) showed strong phylogeographic break in relation to groups I and II, this mean that mean that the Bayesian clustering analysis found

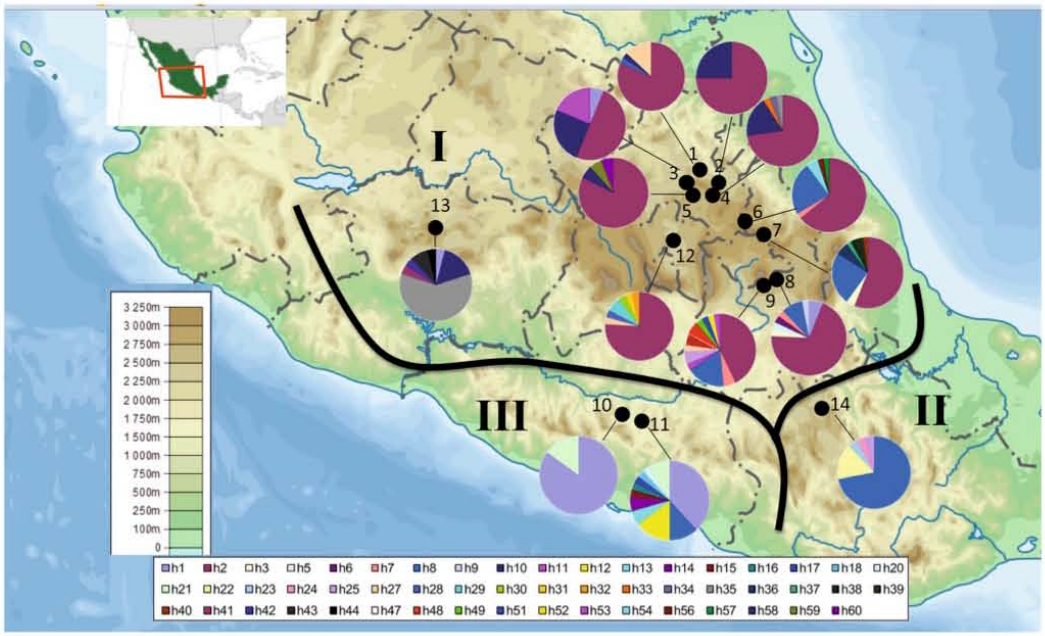


**Fig. 1** Sampled populations in Mexico and genetic structure inferred using the mtDNA (*COI* gene) sequence of *Trichobaris soror*, overlapped in a relief map. Black dots represent populations (codes indicated in Table 1). *Pie charts* depict frequency of haplotypes per population. Geographical clustering, by STRUCTURE and SAMOVA, is indicated by *Roman numerals*

deltaK peaks at $K = 2$ and $K = 3$ (Fig. S3, Supplementary Material).

The same numbers of groups were revealed by the SAMOVA analysis. The highest significant values of population differentiation were found for $K = 2$ (I + II and III) and $K = 3$ (I, II and II) clusters (Table 3). The hierarchical AMOVA analysis shows that 13.02 % of the genetic variance was found among populations when $K = 2$ and 16.46 % when $K = 3$ (Table 3). The differentiation among regions ($\Phi_{ct}$) and within populations ($\Phi_{st}$) are similar in both clusters, but the differentiation among populations within regions ($\Phi_{sc}$) is lower when $K = 3$ ($\Phi_{sc} = 0.11642$) than $K = 2$ ($\Phi_{sc} = 0.24916$; Table 3).

## Haplotype network and phylogenetic reconstruction of *Trichobaris soror*

The haplotype network for *T. soror* also indicated the existence of three groups (Fig. 2; and Figure S4: Supplementary Material). The connection with the outgroup

**Table 3** Hierarchical analysis of molecular variance (AMOVA) based on sequences obtained for cytochrome c oxidase subunit I (*COI*) of *Trichobaris soror*

| *K* | Source of variation | d.f. | Sum of squares | Variance components | Percentage of variation | *P* value | ϕ |
|---|---|---|---|---|---|---|---|
| 2 | Among Regions ($\Phi_{ct}$) | 1 | 718.07 | 7.89106 | 82.65 | 0.00 | 0.8266 |
| | Among populations within regions ($\Phi_{sc}$) | 12 | 160.711 | 0.4126 | 4.32 | 0.00 | 0.2492 |
| | Within populations ($\Phi_{st}$) | 399 | 496.098 | 1.24335 | 13.02 | 0.00 | 0.8698 |
| | Total | 412 | 1374.879 | 7.5532 | | | |
| 3 | Among Regions ($\Phi_{ct}$) | 2 | 811.411 | 6.146 | 81.7 | 0.00 | 0.8137 |
| | Among populations within regions ($\Phi_{sc}$) | 11 | 67.369 | 0.05455 | 2.17 | 0.00 | 0.1164 |
| | Within populations ($\Phi_{st}$) | 399 | 496.098 | 1.24335 | 16.46 | 0.00 | 0.8354 |
| | Total | 412 | 1374.879 | 7.5532 | | | |

*K* = number of groups obtained by STRUCTURE, d.f. = degrees of freedom, ϕ = differentiation index

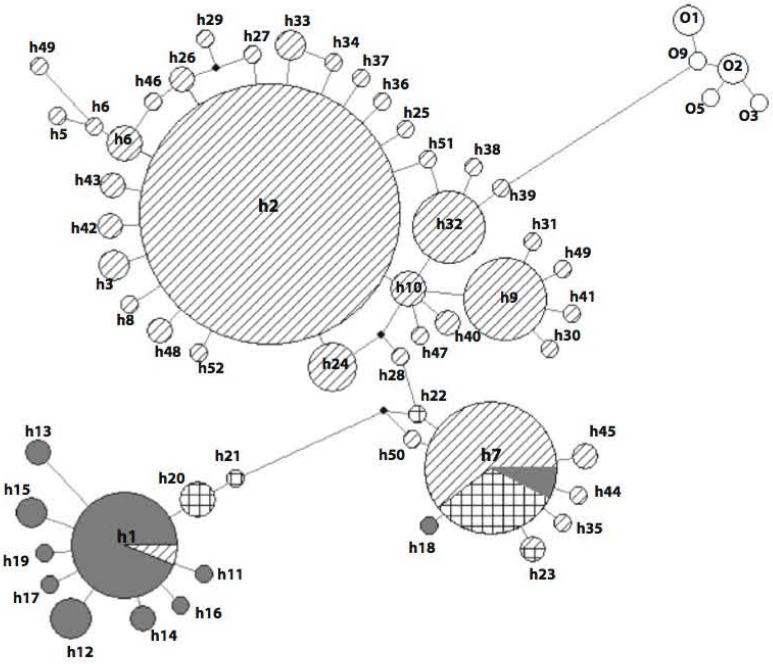


**Fig. 2** *Trichobaris soror* haplotype network based on mtDNA (*COI* gene). Circles size is proportional to haplotype frequency. Proportions of theme within each *pie chart* are representative of the relative number of that haplotype from each region (*backward diagonal* TVB; *cross* SMS, *solid gray* BB and *solid white* outgroup). Numbers on lines represent mutational steps. Haplotypes O1-O5 are from *T. compacta*, the outgroup

haplotypes (*T. compacta)* is through 30 mutational steps, connected with the most frequent haplotypes in the western Transmexican Volcanic Belt. The evolutionary model that best fits the *COI* sequence changes of *T. soror* was GTR + I+G (A = 0.3219, C = 0.1515, G = 0.1336 and T = 0.3931; gamma = 0.2490). This model was applied in constructing the Bayesian inference trees.

Phylogenetic analyses also identified three distinct lineages supporting our genetic structure analysis (Fig. 3). Lineage I includes 34 haplotypes distributed in the Transmexican Volcanic Belt (TVB), lineage II includes 8 haplotypes distributed mainly in the Sierra Madre Sur region (SMS), and lineage III includes 11 haplotypes distributed mainly in the Balsas Basin region (BB). The Bayesian tree topology was similar to the Maximum parsimony and Neighbor joining trees (Figs. S5 and S6, Supplementary material) and shows that haplotypes of the SMS are more related to haplotypes from the TVB (Fig. 3). The outgroup *T. compacta* split from the ingroup 1.80 ± 0.5 million years ago (Mya). Estimated divergence time for the TVB + SMS and BB clades was 1.16 ± 0.25 Mya. The divergence time for the SMS and TVB clades was dated at 0.4 ± 0.2 Mya (the tracer files of this analysis are in Supplementary Material figures S7–S9).

### Historical demography

Neutrality statistics (Tajima's *D* and Fu and Li' *F*) were significantly negative in only the Transmexican Volcanic Belt data ($D$, $p < 0.01$; $F$, $p < 0.002$), suggesting a rapid population expansion. Mismatch distribution analysis was consistent with model of sudden population expansion for the Transmexican Volcanic Belt region (Fig. S10, Supplementary Material).

### Geometric morphometric analysis

The canonical variance analysis shows that the *aedeagus* of weevils from Rancho de Colores (RC) are different shape from all other populations (Fig. 4).

## Discussion

The genetic analysis of *Trichobaris soror*'s populations reveals the existence of three subdivisions in mitochondrial DNA sequences. The phylogeographic pattern of this weevil is explained related to geographical subdivisions (i.e., the geological provinces TVB and SMS are separated by a discontinuity in altitude) as well by the association

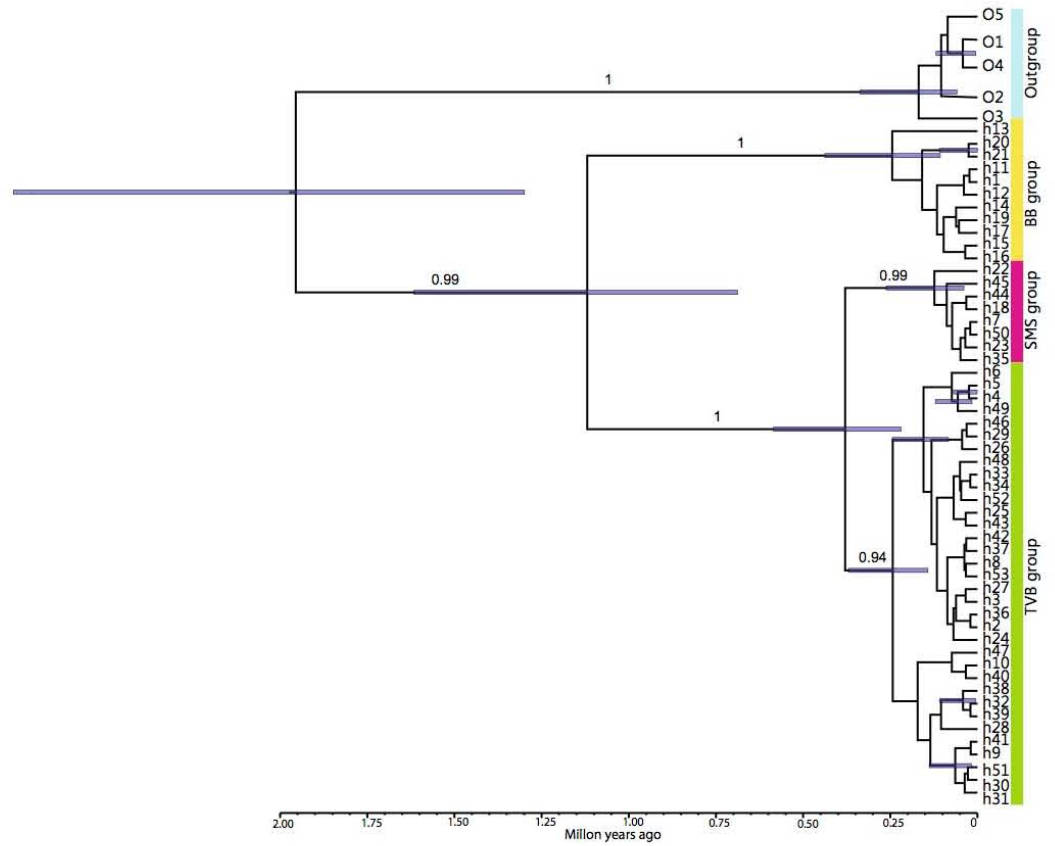


**Fig. 3** Bayesian genealogy for 53 mtDNA haplotypes (*COI* gene) of *Trichobaris soror*, rooted using a sister species (*Trichobaris compacta*) as outgroup. Node support is shown by bootstrap values. *Node bar* is the standard deviation of the age calibrated with a fixed rate of substitution (2 %). Regions where the haplotypes were sampled: Transmexican Volcanic Belt (TVB group), Sierra Madre Sur (SMS group) and Balsas Basin (BB group)

with a host plant (i.e., the recent population expansion of TVB group and the unusual high frequency of haplotype 2 is explained in terms of host interaction). Finally, based on morphometric and genetic analyses, we suggest that populations of *Datura stramonium* from the BB group are parasitized by another, not previously reported, species of *Trichobaris*.

In support of the three groups of *T. soror* detected by the genetic structure analysis, these are distinguished in the haplotype phylogeny and in the haplotype network. These groups are the Transmexican Volcanic Belt (TVB group), Sierra Madre del Sur (SMS group), and Balsas basin group (BB group). Separation between the TVB and SMS *T. soror* groups is estimated from ca. 0.4 mya; the difference in altitude and climate between both regions may explain the distribution of *T. soror*, which is restricted to temperate zones, like its host plant (*D. stramonium*) (Hernández 2009). A similar phylogeographic pattern has been reported for other species of beetles (Anducho-Reyes et al. 2008; Sánchez-Sánchez et al. 2012). It appears that the temperate flora and fauna have been similarly affected by changes in climate and/or topography of the TVB and SMS. Haplotypes found in Balsas Basin split ca. 1.1 mya from haplotypes of *T. soror* from TVB + SMS (Fig. 3). Since the basin formation is estimated 30 mya, from late Eocene to middle Oligocene (Vega et al. 2006), this divergence may represent an speciation event in isolation of *Trichobaris* as

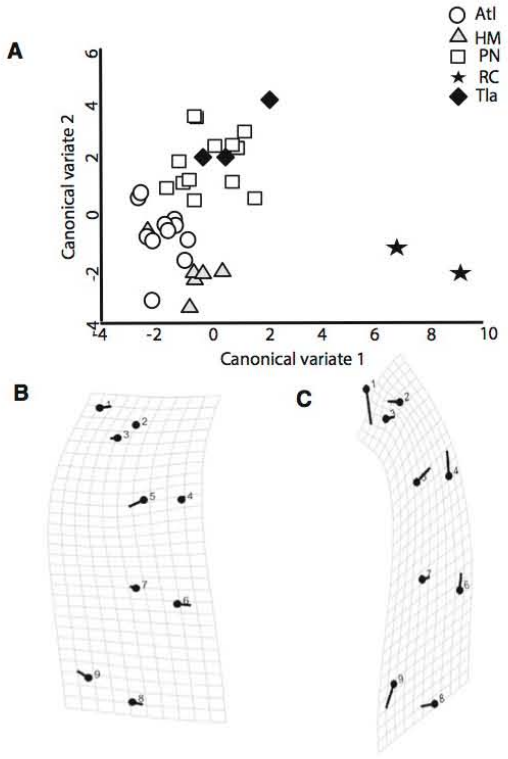


**Fig. 4** **a** The Canonical variate analysis of *Aedeagi* from five populations of the weevil *Trichobaris soror* sampled from *Datura stramonium*'s fruits in Mexico. Individuals from Rancho de Colores (*stars*) are separated from all others: Atlixco (*circles*), Panzacola (*triangles*), Patria Nueva (*squares*), and Tlaxcala (*diamonds*). **b** Transformation grid of CV1 describe the shape of *T. soror's* aedeagi, whereas **c** CV2 describes the shape of weevils found in RC population

*D. stramonium* expanded its distribution or rather an opportunistic change of host by another species of *Trichobaris*.

Historical demography of *T. soror* suggests a recent population expansion of TVB group (Table 4), similar to that found for *Dendroctonus mexicanus* and *D. approximates* in the same region (Anducho-Reyes et al. 2008; Sánchez-Sánchez et al. 2012). This spatial population expansion model suggests that this occurred in the TVB through dispersal as the species extended its distribution range in an east–west direction, consistent with the formation of the TVB (Cevallos-Ferriz and Gonzáles-Torres 2005). This model fits *Dendroctonus* historical demography (Anducho-Reyes et al. 2008; Sánchez-Sánchez et al. 2012) where the major cause of genetic diversity is consistent with dispersal to the new range (Anducho-Reyes et al. 2008). In *T. soror*, population expansion may result from its coevolutionary interaction with *D. stramonium*. We propose two alternative explanations that differ in the degree of synchrony of both demographic expansions. First, the habitat of the plant populations may have expanded due to the emergence of TVB and as a consequence weevil populations expanded simultaneously. Second, the weevils once specialized to *D. stramonium* dispersed along the host plant distribution, resulting in a population expansion. Both scenarios require phylogeographic and demographic signature data for the host plant and should be further explored.

The high frequency of one haplotype can result from a long-term association with one particular host, leading to reductions of genetic variation and ultimately the loss in the ability to use alternative hosts (Kelley and Farrell 1998; Kelley et al. 2000; Thompson 1994, 2005). Excluding two populations (RC and Xo, where $F_{ST}$ values are higher compared to the rest of populations; Table 1), *T. soror* has lower values of population differentiation when compared with *Dendroctonus mexicanus*, a generalist beetle. The high genetic diversity of generalist species is thought to be associated with moderate effects of environmental factors on the demography and distribution of the species, thereby allowing the origin and maintenance of mutations through generations (Hewitt 2000).

Haplotypes from the BB group shows a deep phylogeographic break from *T. soror*. In fact, according to the geometric morphometric analysis, genetic analysis, and Barber's descriptions (1935), this group seems to belong to *Trichobaris mucorea*, a species reported parasitizing *Datura wrightii* and other solanaceous plants such as *Nicotiana attenuata* (Barber 1935; Diezel et al. 2001).

**Table 4** Tajima's *D* and Fu and Li's *F* tests of *Trichobaris soror* by region with significant geographical association

| Region | *D*'s Tajima | *F*'s Fu and Li | $\theta$ | *M* | $\tau$ |
|---|---|---|---|---|---|
| Transmexican Volcanic Belt (TVB group) | −2.1655* | −3.5596** | 0.985 | 0.644 | 3.202 |
| Balsas Basin (BB group) | −1.2872 | −0.1038 | | | |
| Sierra Madre Sur (SMS group) | 0.7298 | −1.2855 | | | |

Expansion parameters, $\theta$ = diversity index, M = migration rate per generation, $\tau$ = time since the population expansion

* $P < 0.01$; ** $P < 0.02$

Finally, this work is the first phylogeographic study of a species of the *Trichobaris* genus, which includes many species that are pests of potato, tomato, and tobacco crops. *T. soror*, as mentioned before, has been only reported in *D. stramonium*; our results set the first step in the understanding of the evolution of *Trichobaris*, and give us the possibility to test if of the phylogeographic pattern of *T. soror* and *D. stramonium* match each other, and how *T. soror* is related with others *Trichobaris* species.

## Conclusion

The phylogeographic pattern of *T. soror* can only be explained in part by the geological history of its distribution zone. The finding of three genetic groups is explained by isolation between the TVB and the SMS regions and by the potential infestation of *D. stramonium* by another species of *Trichobaris* in the BB region. Further research will determine if genetic diversity and historic demography of *T. soror* is tightly linked to its host plant *Datura stramonium*.

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**Compliance with ethical standards**

**Conflict of interest** Authors do not have any financial relationship with the organization that funded the research. The authors declare that they have no conflict of interest.

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**Artículo de investigación 2.**

# Phylogeography of the generalist weevil *Trichobaris compacta*: a seed predator of *Datura* genus

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## Abstract

Genetic variation and structure of populations are expected to differ between host specialist and generalist species of herbivorous insects. Due to local adaptation, genetic variation would be lower in a host specialist species and its geographic structure will be greater than in generalist species. This study aimed to compare the phylogeography of *Trichobaris compacta,* a host generalist weevil species of plants in the genus *Datura,* and the host specialist *T. soror*. For *T. compacta*, we amplified 663bp of the COI gene in a sample of individuals from different localities, a total of 49 haplotypes were found in 232 samples. We found four groups in the analysis of population structure associated to the distributional area, neither associated to one host plant species. *T. soror* and *T. compacta* did not differ in the amount of genetic variation according to what is expected among specialist and generalist insects, however the rarefaction curves showed that more populations of *T. compacta* should be need to make a more accurate comparison.

## Resumen

La variación y estructuración genética se espera sean distintas entre especies de insectos herbívoros especialistas y generalistas respecto de sus plantas huésped. Debido a la adaptación local de planta huésped, la variación genética y el patrón de estructuración geográfica se espera sean menores en herbívoros especialistas que en los generalistas. En este estudio comparamos la filogeografía de *Trichobaris compacta,* un curculiónido generalista de especies del género *Datura,* y *T. soror,* una especie especialista. Para ello, se amplifico el gen COI (663 pb) en individuos de *T. compacta*

muestreados de distintas localidades. Se encontraron un total 49 haplotipos en 232 muestras. Los resultados indican que la estructura genética poblacional se divide en cuatro grupos asociados al área de distribución y no así a la planta huésped. No se detectaron diferencias en la cantidad de variación genética entre especies de *Trichobaris*. Sin embargo, las curvas de rarefacción mostraron que se requieren más poblaciones de *T. compacta* para hacer una comparación más precisa.

**Keywords:** Host-plant interaction *Datura* Phylogeography *Trichobaris* Weevil

## Introduction

Weevils are one of the most successful group of phytophagous insects (Girdmaldy and Egel 2005). With approximately 60 000 species, this great diversity is due to their interaction with angiosperm plants (Oberprieler, *et al.,* 2007; Farrell 1998; Mckenna *et al.,* 2009). The hypothesis proposes that in mono- and/or oligophagous herbivores, associations with host plants may contribute to the formation of genetically distinct host races and ultimately to speciate (Erich & Raven, 1964). Specialization in any form is often regarded as something advanced and efficient, many conferring short-term advantages but increasing long term vulnerability. Specialization is some times becomes a prelude to extinction but in other cases evolutionary change that constitutes extreme specialization enables organisms to exploit new niches and to radiate and fill a new adaptive zone (Newton, 2003).

It is supposed that specialization reduces genetic variation of insect populations due to selection for more efficient resource use. Some studies show substantial differences in the amounts of genetic diversity in mtDNA between generalist and specialist sister species, revealing that specialist species might have smaller effective population sizes (Kelley *et al.,* 2000). However small effective population size and patchy distributions of host plants can potentially result in increased isolation among populations, potentially having macro-evolutionary consequences for lineage diversification (Forister, *et al.,* 2012). Since there are no obvious general trends toward specialization for many phytophagous insects, the analysis of the phylogeographic component of genetic variation can help us to understand the role of insect-plant interactions in the process of population differentiation.

The phylogeographic pattern of generalist and specialist herbivores indicates that the former offer a more detailed history of the area of distribution in contrast with specialists whose phylogeographic pattern is constrained by the host plant (Aoki, *et al.,* 2009). Comparing the intraspecific

phylogeographical patterns among taxa over the same area searching for congruent geographical patterns of genetic variation, can indicate the influence of common historical factors (Avise, 2004) and rule out the effects of specialization on the amount genetic variation.

To date few phylogeographic studies of specialist and non-specialist weevils have been carried out in North America (but see Kuester, *et al.,* 2012; Barr, *et al.,* 2013; Sánchez-Sánchez *et al.,* 2012; Anducho-Reyes *et al.,* 2008) despite that Quaternary events have had a great impact on biodiversity of this region (Williams *et al.,* 2004). Particularly, in Mexico where the current topography has been recently emerged (Morán-Zenteno, 1984). Here, it has been found that specialist weevils like cotton weevil, shows mitochondrial haplotypes restricted to its host plant, whereas the evolutionary history of *Dendroctonus pseudosugae* is in agreement to its host plant (Douglas-fir) (Ruiz, *et al.,* 2010). This is not the case for *Dendroctonus mexicanus* and *D. approximatus* whose evolutionary history seems to be better explained by the sequence of colonization events on their current range of distribution. These species did not show genetic variation restricted to a host plant, but in both cases high levels of haplotype diversity are reported suggesting a recent population expansion (Anducho-Reyes, *et al.,* 2008; Sánchez-Sánchez, *et al.,* 2012)

Here we analyzed the genetic variation of the widely distributed weevil, *Trichobaris compacta* a generalist herbivore that feeds on seeds of *Datura* species, in order to compare its phylogeographic pattern with that of *T. soror*, a host-specialist weevil of *D. stramonium*. For this propose, we used a fragment of the mitochondrial COI gene to determine: (1) population differentiation and structure, (2) historical demography and (3) genealogical relationships to know if *T. compacta* exhibits some host specialization among *Datura* species.

## MATERIALS AND METHODS

### Study site and insect collection

The distribution of *Trichobaris compacta* is reported on the south west of USA (Barber, 1935) feeding on *Datura* species. In our group of research, we have found *Trichobaris compacta* in some populations of *Datura* plants along the Pacific coast. Based on both data we design the sampling covering at least 20 plants of each population to sample the insects. Adults insects collected on plants were preserved in absolute alcohol for posterior analysis. Several fruits per plant were

collected. In order to avoid sampling relatives, we performed genetic analysis taking only one weevil from each fruit. A total of 29 locations along the host plant distribution were sampled (Table 1; Figure 1).

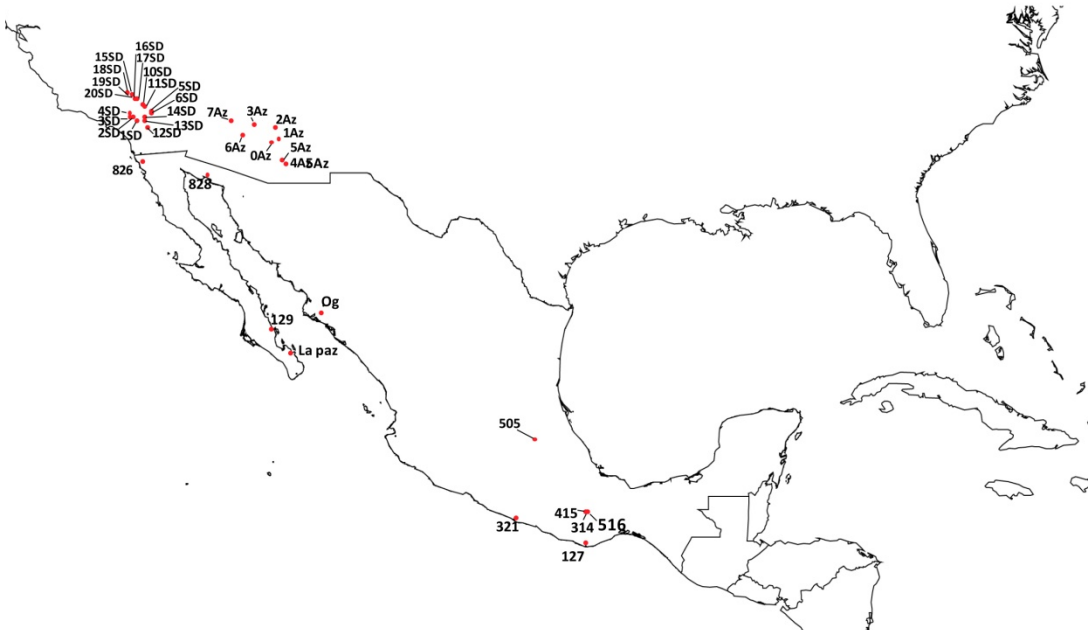



Figure 1. Sampled location where *Trichobaris compacta* were collected upon several *Datura* plant species (see Table 1).

**DNA extraction, PCR and sequencing**

Each insect was frozen in liquid nitrogen (-196 °C) and then macerated with a micropestle. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen$^{TM}$) according to the manufacturer's protocol for animal tissue. We then amplified a region of 663bp nucleotide protein coding region of the mitochondrial gene cytochrome c oxidase I (COI) by polymerase chain reaction (PCR) using the following primers: COA3107 (TCT ATT ARD GGD GAD GCD CTA TCT TG) and COS2183N (CAR CAY YTA TTY TGR TTY TGR TTY TTY GG) (Sota, *et al.*, 2004).

The polymerase chain reactions (25.5 µl in volume) contained each 1 µl DNA (20 nM), 1 µl each primer (10 mM), 0.2 µl of taq polymerasa (GoTaq Promega), 5µl of Buffer 5x, 0.5 µl each nucleotide (10 mM), 3µl $MgCl_2$ (25 mM) and 8.6 µl $H_2O$. The thermal cycling conditions were as follows: an initial period of 5 min at 95 ºC, followed by 35 cycles of 60s at 95°C, 1.2 min at 55°C, and 60s at 72 °C, with a final extension for 7 min at 72 °C. PCR products were sequenced at

Washington University using an ABI 3730xl sequencer (Applied Biosystems™). All nucleotide sequences obtained were compared, edited manually with Sequencher™ 4.7 software, and aligned with MAFFT (Katoh & Standley, 2013).

**Genetic variation analysis and population structure**

To compare the levels of diversity of *T. compacta* populations (Table 1), we estimated the total number of haplotypes, mutations, segregating sites ($S$), nucleotide diversity ($\pi$), haplotype diversity ($h$) and theta ($\theta$), using the Nei's (1987) equations implemented in version 5.1 of the DNAsp program (Rozas, 2003).

The differentiation index ($F_{ST}$) between populations (Table 2) was estimated using Arlequin ver. 3.11 (Excoffier *et al.,* 2005). Population structure was estimated by two methods. First, we used a Bayesian clustering analysis in BAPS (Corander *et al.,* 2005; Corander *et al.,* 2008) for linked loci (Corander & Tang, 2007), the maximum likelihood value was used to determine the number of genetic groups. We performed a mixure analysis wit $K$ values from 2 to 12 and with 3 iterations each one. Then we used the output file to performed the admixure analysis. The minimum size of populations to be taken into account was set to 1, while 100 iterations were applied to estimate the admixture coefficients of the individuals, 10 of reference individuals from each population were used and, finally, 100 iterations were performed in order to estimate the admixture coefficients of the reference individuals. Second, we used a SAMOVA approach (Spatial Analysis of Molecular Variance; Dupanloup & Excoffier, 2002), which defines populations that are geographically homogeneous and maximally differentiated from each other. The method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences among groups of populations. Simulations have shown that the SAMOVA algorithm does indeed find maximally differentiated groups, especially when data derive from a single locus.

**COI haplotype network and phylogenetic analysis**

To visualize the frequency and distribution of haplotypes we constructed an un-rooted haplotype network, using the Median-joining algorithm; the analysis was performed in Network v. 4.6.1.1.

(Bandelt *et al.*, 1999), in order to visualize the distribution of COI haplotypes on the host plant, they were colored according to the host plant where they were collected (Figure 3).

A haplotype phylogeny was built by Bayesian Inference in BEAST v.1.4.7 (Drummond & Rambaut, 2007). The parameters of MCMC runs were 30 million generations, sampling every 1000 generations and discarding the first 10,000 trees as burn-in. Using a GTR+I model of nucleotide substitution and a fixed substitution rate of 2 % per million years for COI, as previously reported for coleopterans (Nakamine & Takeda, 2008). In order to identify ancestral and derived haplotypes in the phylogeny, the trees were rooted using *Trichobaris soror*. The samples were summarized in the maximum clade credibility tree (BEAST v.1.4.7 package software; Drummond & Rambaut, 2007). The phylogeny was visualized and edited with Figtree 1.0 (Rambaut, 2006).

**Historical demography**

Assuming neutrality, we estimated the Fu and Li's *F* (1993) and Tajima's *D* statistics in order to infer population historical demography. The Tajima's *D* statistic is expected to be negative when genetic structure has been influenced by rapid range expansion, positive when the population has passed through a bottleneck, and zero when there is equilibrium between mutation and drift (Tajima, 1989). To compare observed frequencies of pairwise differences with those expected under a model of demographic expansion, mismatch distributions were generated using DNASP v.4.10 (Rozas, 2003). A multimodal distribution is expected when there are no changes affecting population size, but unimodal distributions are expected when sudden demographic expansions have occurred (Rogers & Harpending, 1992).

**RESULTS**

**COI variation and population structure**

We sequenced 663 bp of COI mtDNA in 232 individuals of *Trichobaris compacta* from 29 localities. We found 49 haplotypes, 33 segregation sites (S), 56 singletons, (Table 1). Sequences without missing bases were submitted to GenBank (KX359683 to KX359723). *T. compacta* populations have high genetic diversity ($h$ =0.709, $\pi$ =004, $\theta$ =0.032). The most abundant haplotype (Co1) is present in 50 % of the total sample (Table 1; Fig. 2).

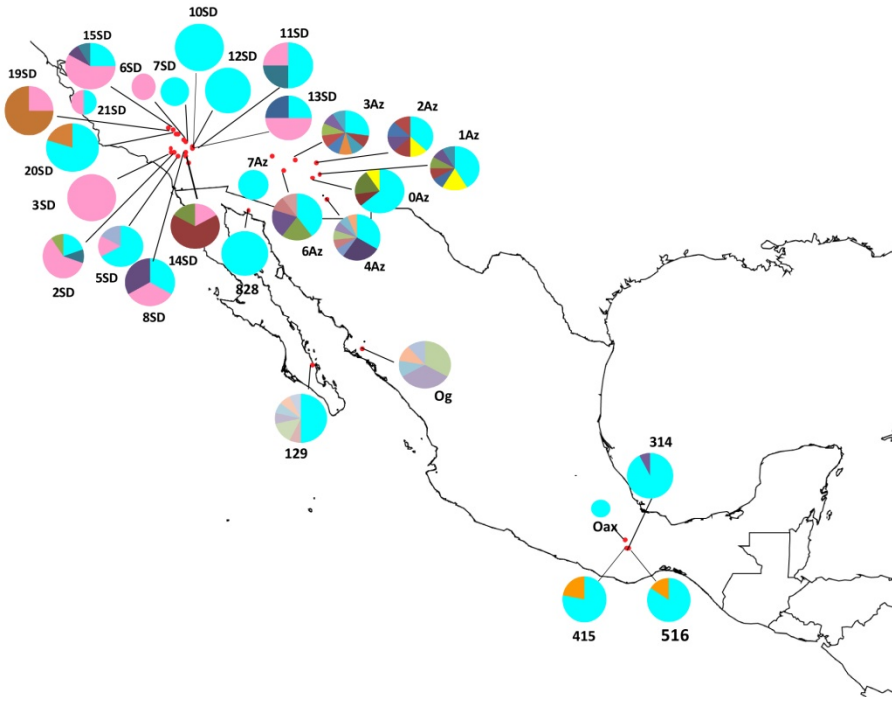
High and significant genetic differentiation among populations was detected. The most differentiated populations were 14SD, 19SD and 3SD with $F_{ST}$ from 0.0761 to 1.0 among them (Table 2). The Bayesian clustering analysis performed with BAPS showed that the maximum value of livelihood is reached at $K$=4 (Figure 3). Whereas with SAMOVA the first higher value of $F_{CT}$ was found at $K$=3, and a second one at $K$=9 (Fig. 1S; Supplementary Material).

Table 1. Populations sampled of *Trichobaris compacta* and genetic diversity values estimated from 663pb of the COI gene. $h$ haplotype diversity, $\pi$ nucleotide diversity and $\theta$.

| Number | State, Country | Locality | Coordinates | # insects | # haplotypes | # mutations | $S$ | Singletons | $h$ | $\pi$ | $\theta$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Arizona,USA | 0Az | 32°35'32.79"N, 110°50'56.54"W | 11 | 4 | 5 | 5 | 2 | 0.600 | 0.002 | 0.002 |
| 2 | Arizona,USA | 1Az | 32°36'50.10"N, 110°49'57.91"W | 12 | 7 | 6 | 6 | 5 | 0.833 | 0.001 | 0.003 |
| 3 | Arizona,USA | 2Az | 32°58'42.19"N, 110°46'8.08"W | 8 | 6 | 5 | 5 | 4 | 0.893 | 0.002 | 0.002 |
| 4 | Arizona,USA | 3Az | 33° 9'21.73"N, 111°46'36.77"W | 10 | 9 | 14 | 13 | 11 | 0.945 | 0.004 | 0.007 |
| 5 | Arizona,USA | 4Az | 32° 3'48.57"N, 110°17'3.42"W | 15 | 8 | 8 | 8 | 7 | 0.848 | 0.002 | 0.003 |
| 6 | Arizona,USA | 6Az | 33°21'50.08"N, 112°37'32.06"W | 10 | 5 | 7 | 7 | 5 | 0.822 | 0.002 | 0.003 |
| 7 | Arizona,USA | 7Az | 33° 5'25.83"N, 112° 2'1.26"W | 2 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 8 | California,USA | 2SD | 33°34'26.50"N, 117°10'52.59"W | 10 | 4 | 3 | 3 | 1 | 0.644 | 0.001 | 0.001 |
| 9 | California,USA | 3SD | 33°45'41.29"N, 117°11'27.19"W | 8 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 10 | California,USA | 5SD | 33°46'4.14"N, 116°19'28.23"W | 6 | 3 | 3 | 3 | 3 | 0.600 | 0.001 | 0.001 |
| 11 | California,USA | 6SD | 33°46'19.52"N, 116°19'53.98"W | 1 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 12 | California,USA | 7SD | 33°35'42.08"N, 116° 5'52.62"W | 1 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 13 | California,USA | 8SD | ____ | 3 | 3 | 5 | 5 | 5 | 1.000 | 0.005 | 0.005 |
| 14 | California,USA | 10SD | 34° 6'52.93"N, 116°27'56.07"W | 7 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 15 | California,USA | 11SD | 34° 8'24.06"N, 116°24'48.14"W | 6 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 16 | California,USA | 12SD | 33° 5'38.23"N, 116°57'47.19"W | 4 | 3 | 2 | 2 | 1 | 0.833 | 0.001 | 0.001 |
| 17 | California,USA | 13SD | 33°29'28.28"N, 117° 3'28.97"W | 4 | 3 | 3 | 3 | 3 | 0.833 | 0.002 | 0.002 |
| 18 | California,USA | 14SD | 33°29'1.49"N, 116°54'45.23"W | 6 | 3 | 6 | 6 | 6 | 0.600 | 0.003 | 0.003 |
| 19 | California,USA | 15SD | 34°10'35.93"N, 116°25'36.47"W | 12 | 4 | 6 | 6 | 4 | 0.636 | 0.002 | 0.003 |
| 20 | California,USA | 19SD | 34°15'18.02"N, 116°26'19.72"W | 4 | 2 | 1 | 1 | 1 | 0.500 | 0.001 | 0.001 |
| 21 | California,USA | 20SD | 33°59'46.45"N, 116°34'43.69"W | 5 | 2 | 1 | 1 | 1 | 0.400 | 0.001 | 0.001 |
| 22 | California,USA | 21SD | 33°57'29.11"N, 116°35'30.66"W | 2 | 2 | 2 | 2 | 2 | 1.000 | 0.003 | 0.003 |
| 23 | Oaxaca,Mexico | 314 | 16 47' 11.61''N, 96 12' 42.34''W | 13 | 2 | 4 | 4 | 4 | 0.154 | 0.001 | 0.001 |
| 24 | Oaxaca,Mexico | 415 | 16 40' 2.60''N, 96 22' 48.98''W | 18 | 2 | 1 | 1 | 0 | 0.366 | 0.001 | 0.000 |
| 25 | Oaxaca,Mexico | 516 | 16 28' 46.10''N, 96 13' 3.51''W | 19 | 2 | 1 | 1 | 0 | 0.281 | 0.001 | 0.001 |
| 26 | Sonora, Mexico | 828 | 32 11' 28.14''N, 114 55' 19.63''W | 9 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 27 | BajaCalifornia, Mexico | 129 | 26 0' 21.46N, 111 20' 35.34"W | 14 | 7 | 17 | 17 | 14 | 0.758 | 0.004 | 0.008 |
| 28 | Oaxaca,Mexico | Oax | 16 55' 11.92''N, 96 23' 6.10''W | 3 | 1 | 0 | 0 | 0 | 0.000 | 0.000 | 0.000 |
| 29 | Sinaloa, Mexico | OG | 25 26' 25.92''N, 108 3' 59.49''W | 9 | 5 | 7 | 7 | 5 | 0.833 | 0.003 | 0.003 |
| | | | Total | 232 | 49 | 57 | 33 | 56 | 0.704 | 0.002 | 0.014 |

Table 2. Population differentiation values *Fst* among sampling sites (at least n=3) of *T. compacta* estimated using 663bp of COI gene. The statistically significant values are highlighted in bold.

| | 0Az | 10SD | 11SD | 12SD | 13SD | 14SD | 15SD | 19SD | 1Az | 20SD | 2Az | 2SD | 314 | 3Az | 3SD | 415 | 4Az | 516 | 5SD | 6Az | 8SD | 854 | OG | 129 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **0Az** | 0 | | | | | | | | | | | | | | | | | | | | | | | |
| **10SD** | -0.02416 | 0 | | | | | | | | | | | | | | | | | | | | | | |
| **11SD** | -0.0425 | 0 | 0 | | | | | | | | | | | | | | | | | | | | | |
| **12SD** | -0.07326 | 0.37778 | 0.33597 | 0 | | | | | | | | | | | | | | | | | | | | |
| **13SD** | 0.02065 | **0.68539** | **0.6555** | 0.0303 | 0 | | | | | | | | | | | | | | | | | | | |
| **14SD** | **0.14712** | **0.59924** | **0.57143** | 0.41779 | 0.49964 | 0 | | | | | | | | | | | | | | | | | | |
| **15SD** | **0.11389** | **0.42943** | **0.40822** | -0.01775 | -0.1134 | **0.45748** | 0 | | | | | | | | | | | | | | | | | |
| **19SD** | 0.176 | **0.93819** | **0.93084** | 0.58333 | 0.33333 | **0.68294** | **0.29412** | 0 | | | | | | | | | | | | | | | | |
| **1Az** | -0.05642 | -0.05027 | -0.06748 | -0.10039 | -0.02647 | 0.07276 | **0.0761** | 0.0889 | 0 | | | | | | | | | | | | | | | |
| **20SD** | -0.06118 | 0.07285 | 0.04 | 0.20561 | 0.54204 | **0.48974** | **0.37126** | **0.84966** | -0.08684 | 0 | | | | | | | | | | | | | | |
| **2Az** | -0.01334 | 0.02778 | 0.00415 | 0.12016 | **0.41782** | **0.4533** | **0.36122** | **0.68248** | -0.04096 | -0.00607 | 0 | | | | | | | | | | | | | |
| **2SD** | **0.13526** | **0.63467** | **0.6156** | 0.09977 | -0.11997 | **0.58798** | -0.05263 | **0.37998** | **0.0851** | **0.55954** | **0.48498** | 0 | | | | | | | | | | | | |
| **314** | 0.03473 | -0.05507 | -0.07216 | 0.23066 | **0.5772** | **0.57773** | **0.43563** | **0.8078** | 0.00465 | -0.02572 | **0.05796** | **0.58072** | 0 | | | | | | | | | | | |
| **3Az** | 0.0197 | 0.01107 | -0.0088 | 0.04554 | **0.27623** | **0.3501** | **0.29915** | **0.50592** | -0.0083 | -0.02136 | -0.06549 | **0.37039** | **0.06603** | 0 | | | | | | | | | | |
| **3SD** | **0.22059** | **1** | **1** | **0.68091** | 0.18644 | **0.76494** | 0.12727 | **0.78082** | **0.14226** | **0.93272** | **0.74026** | 0.11581 | **0.83622** | **0.54633** | 0 | | | | | | | | | |
| **415** | **0.07638** | 0.07692 | 0.0597 | **0.37173** | **0.66068** | **0.66556** | **0.51155** | **0.86863** | **0.03714** | 0.10087 | **0.14363** | **0.6584** | 0.08211 | **0.12478** | **0.88655** | 0 | | | | | | | | |
| **4Az** | 0.02723 | 0.03789 | 0.01987 | 0.14849 | **0.43646** | **0.4826** | **0.38521** | **0.66746** | 0.01206 | 0.02001 | 0.03292 | **0.48346** | **0.08025** | 0.06514 | **0.68592** | **0.14364** | 0 | | | | | | | |
| **516** | **0.07766** | 0.02026 | 0.00382 | **0.40377** | **0.70064** | **0.68948** | **0.52753** | **0.8906** | **0.03843** | 0.08123 | **0.13808** | **0.68056** | 0.04984 | **0.12247** | **0.90851** | -0.04302 | **0.13506** | 0 | | | | | | |
| **5SD** | -0.04722 | 0.02778 | 0 | -0.07652 | 0.30594 | **0.44898** | 0.20728 | **0.69017** | -0.06574 | -0.01538 | 0.02041 | 0.36701 | 0.02447 | 0.01033 | **0.76494** | 0.12973 | 0.05357 | 0.12131 | 0 | | | | | |
| **6Az** | 0.00871 | 0.00517 | -0.01538 | 0.0958 | **0.38251** | **0.43346** | **0.34857** | **0.63461** | -0.02115 | -0.02041 | -0.03158 | **0.45333** | **0.05049** | -0.00286 | **0.67747** | **0.12121** | 0.02444 | **0.11364** | 0.0129 | 0 | | | | |
| **8SD** | -0.10752 | **0.3** | 0.25 | -0.12442 | 0.03943 | 0.05063 | 0.01114 | 0.44476 | -0.14006 | 0.14207 | 0.12152 | 0.20983 | 0.27113 | 0.05063 | 0.58692 | **0.41084** | **0.18227** | **0.44372** | 0 | 0.11117 | 0 | | | |
| **854** | 0.0028 | 0 | 0 | 0.44493 | **0.73134** | **0.64427** | **0.46535** | **0.94901** | -0.02574 | 0.12621 | **0.06494** | **0.66677** | -0.03084 | 0.04129 | **1** | 0.1032 | 0.06513 | 0.04436 | 0.07216 | 0.03656 | **0.37931** | 0 | | |
| **OG** | **0.46412** | **0.81133** | **0.79888** | **0.74021** | **0.75582** | **0.75984** | **0.76546** | **0.8144** | **0.35755** | **0.7698** | **0.74243** | **0.80148** | **0.81345** | **0.6672** | **0.86041** | **0.85265** | **0.75126** | **0.86327** | **0.75483** | **0.73341** | **0.69347** | **0.8319** | 0 | |
| **129** | **0.03435** | -0.00257 | -0.02038 | 0.03435 | **0.25748** | **0.33026** | **0.28257** | **0.47795** | 0.00977 | -0.04282 | 0.0384 | **0.33541** | 0.05164 | **0.06126** | **0.50383** | **0.10031** | **0.08659** | **0.09634** | 0.00242 | **0.05068** | 0.04532 | 0.02374 | **0.6656** | 0 |

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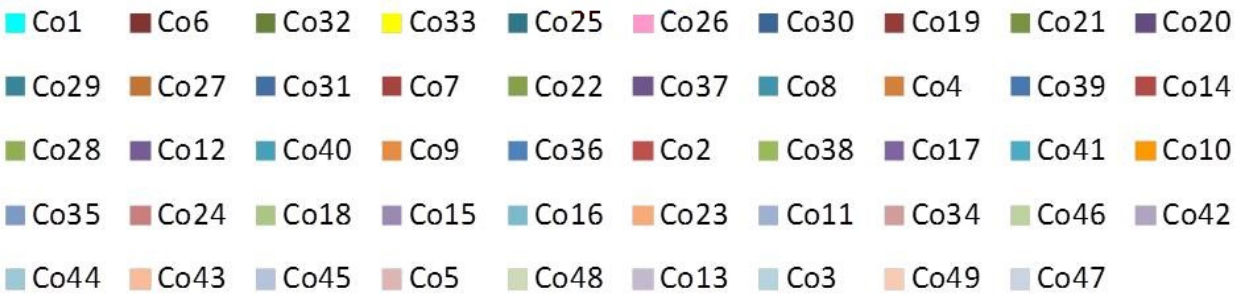
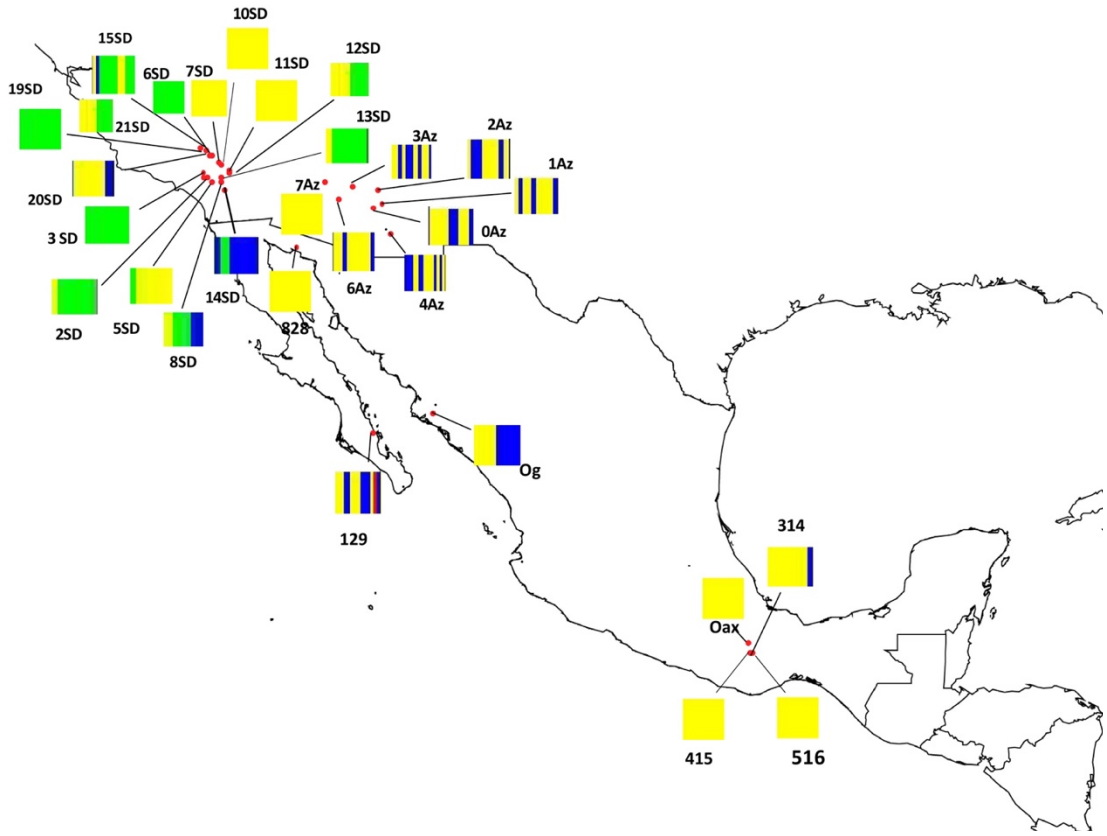



177

Figure 2. Haplotype distribution of gene COI (663bp) from *Trichobaris compacta* in sampled localities. Red dots represent localities, pie charts depict frecuency of haplotypes and size of circle is proportional to sampled size (see Table 1 for locality codes and sample size).




178

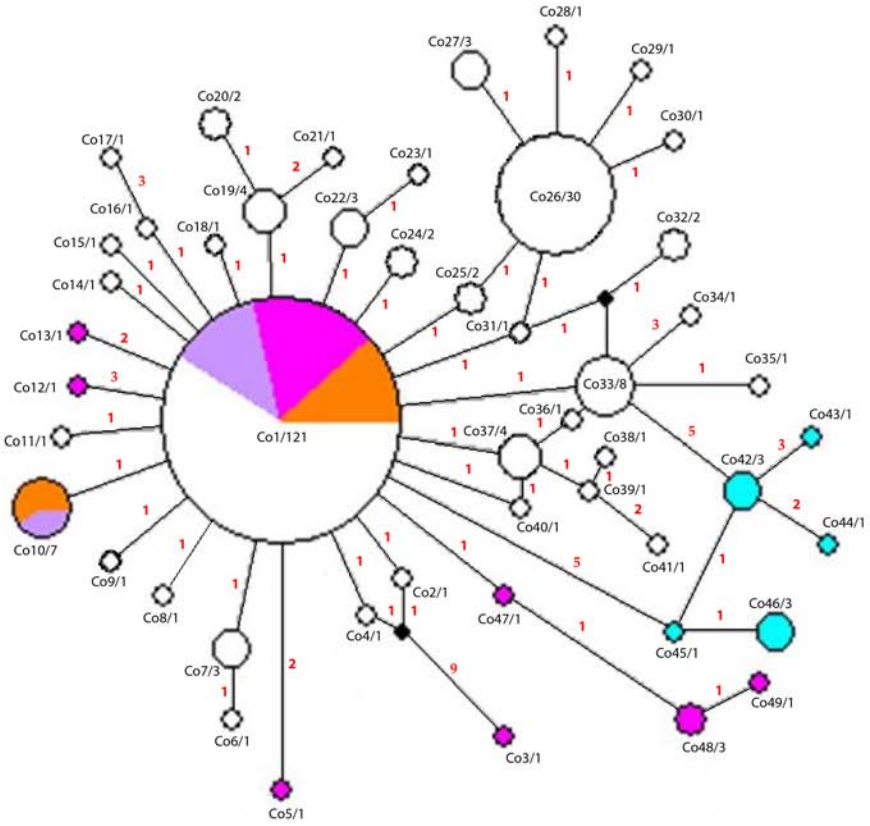
179 Figure 3. Population structure of *Trichobaris compacta.* Each color represent a genetic group
180 determined by Bayesian clustering using *COI* gene (663bp). The localities are described on table 1.

181

182 **COI haplotype network and phylogenetic analysis**

183 From a sample of 232 insects 49 haplotypes were found. The most frequent and shared haplotypes
184 among populations were Co1 (52% of total sampling), Co26 (13%), Co33 (3%), and Co10 (3%)
185 (Fig. 4). From these, Co1 and Co10 were sampled in more than one host-plant (*D. wrightii*, *D.*
186 *discolor*, *D. inoxia* and *D. pruinosa*), while seven haplotypes were found exclusive in *D. discolor*
187 and five in *D. reburra*; a total of 35 were found in *D. wrightii*.

188




189

Figure 4. Haplotype network of *Trichobaris compacta*, calculated using variation of gene COI (663pb) by the "Median joining" algorithm. Black dots represent vectors and red numbers the number of mutational changes. The colors represent the species of plant where they were colected: withe-*Datura wrightti*, pink-*D.discolor*, purpure-*D.inoxia*, turquise-*D.reburra* and orange-*D.pruinosa*.

190

191 The phylogenetic analysis with COI haplotypes do not support the formation of any clade of *T.*
192 *compacta.* The species is well supported with posterior probability of 1.0, separated from *T. soror*
193 aproximately 1.75(±1) m.a. with a recent divesification (0.5 ± 0.25 m.a.) (Fig. 5).

194

195

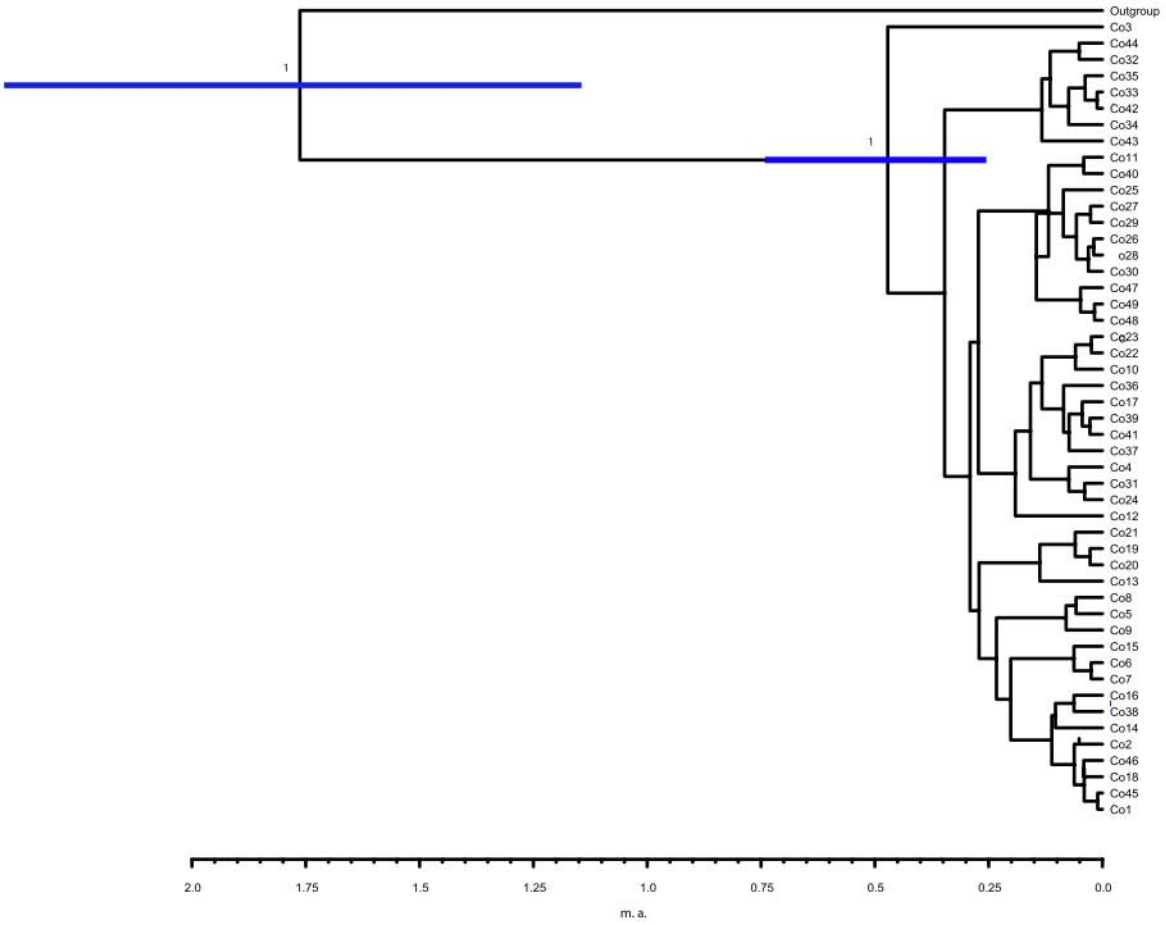



Figure 5. Phylogeny of *Trichobaris compacta* built with COI gene (663bp) haplotypes, by Bayesian inference. Numbers at nodes show the posterior probability. Calibrated with 2% of divergence reported for COI gene, the bar represents the standard error of the estimation.

**Historical demography**

The analysis of historical demography shows a unimodal distribution of mismatches (Fig. 5), which is characteristic of population expansion. This result is congruent with the negative values of Tajima's *D*= -2.69330 p<0.001 and Fu and Li' *F*= -5.10607 p<0.02.

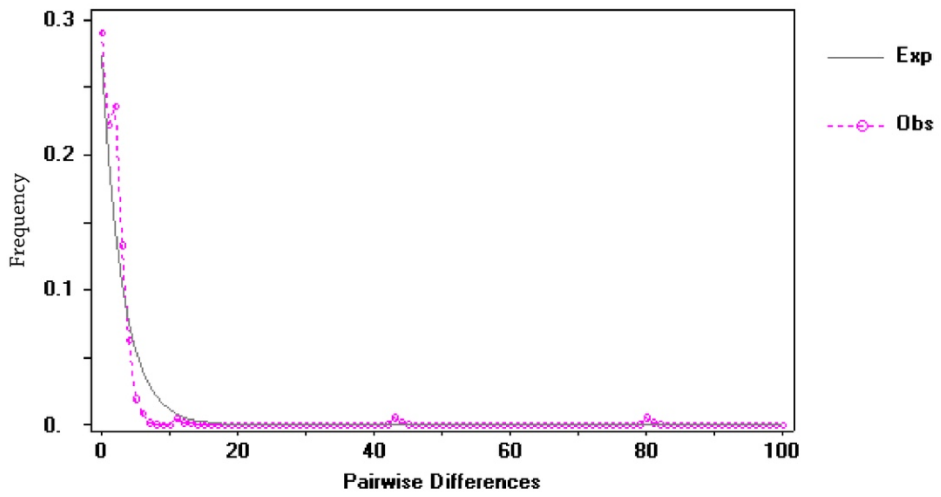



Figure 5. Historical demography of *Trichobaris compacta.* Mismatch distribution calculated with the 663bp of COI gene sequence.

**Comparison of genetic diversity, haplotype network and phylogenetic tree between *T. compacta* and *T. soror.***

For the comparison we used data previously published of *T. soror* (De-la-Mora, *et al.,* 2015), excluding populations RC and Xo that appear to be another species. The number of COI haplotypes in *T. compacta* is 49 (n= 232) whereas in *T. soror* is 51 (n= 369). Genetic diversity is higher in *T. compacta* ($h$ = 0.709, $\pi$ = 0.004, $\theta$ = 0.031) than in *T. soror* ($h$ = 0.663, $\pi$ = 0.005, $\theta$ = 0.021). In order to make a proper comparison the rarefaction analyses were performed for *T. soror* and *T. compacta* with the software PAST ver. 3.13 (Hammer et al., 2001), using the $S_{obs}$ (Mao's tau) as a measure of haplotype richness, obtained after resampling 100 times with replacement. Here the haplotype richness tends to be larger in *T. compacta* that in *T. soror* (Figure 6).

Haplotype networks of both species show a start-like pattern, is indicative of a recent population expansion. And both are negative and statistically significant for neutrality test. In *T. compacta* Tajima's *D*= -2.69330 and Fu and Li'*F*= -5.10607 whereas in *T. soror* Tajima's *D*= -2.226 and Fu and Li'*F*= -6.10.

The phylogenetic tree of *T. compacta* shows no clades with high support contrary to *T. soror* that shows two cades one distributed on the Transmexican volcanic belt and the other on the Sierra Madre Sur.

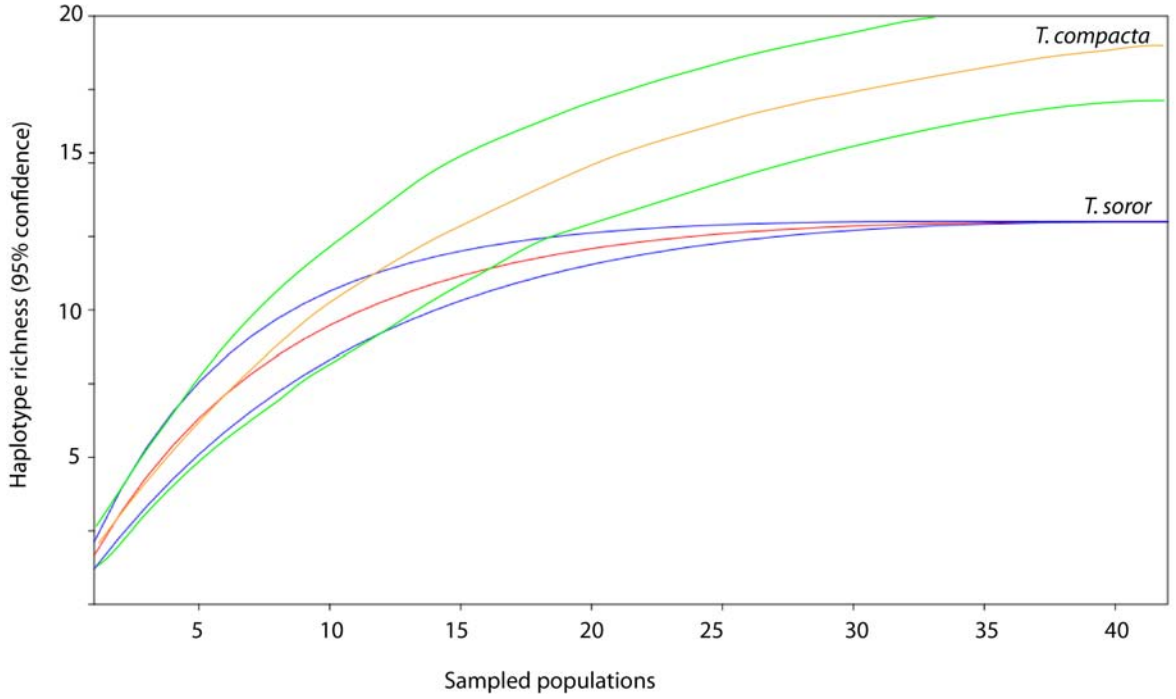



Figure. 6. Smooth richness accumulation curves (sample–based rarefaction curves) for *Trichobaris compacta* and *Trichobaris soror*. In both cases the mean value (and the standard deviation) of the haplotypes richness for increasing sampling efforts.

## DISCUSION

### Genetic variation of generalist and specialist weevils

We expected that the genetic variation of the generalist to be greater than of the specialist weevil (Kelley *et al.,* 2000; Forister, *et al.,* 2012). In this case the genetic variation of *T. compacta* was slightly higher than in *T. soror*. In general, generalist weevils showed high levels of genetic variation. For example, in the generalist seed predator *Curculio sikkimensis,* 41 COI (921bp) haplotypes were found among n=115 with *h*= 0.9333, and $\pi$=0.004 (Aoki & Murakami, 2009). In the generalist cotton weevil, *Anthonomous grandis,* in which 66 haplotypes were found for n= 115 with *h*= 0.836 and $\pi$=0.014 (Kuester, *et al.* 2012). Finally, in the case of the generalist bark beetle *Dendroctonus mexicanus* in which 53 COI (254bp) haplotypes in n= 173 with *h*=0.849 and $\pi$= 0.015 (Anducho-Reyes, *et al.*, 2008). Howhever some reports show higher levels of genetic variation in specialist weevils, such as bark beetle *Dendroctonus pseudotsugae* where n= 331 had 136 COI haplotypes (550bp), *h*= 0.943 and $\pi$=0.022) (Ruiz, et al. 2010).

Others studies are not directly comparable because these use more mitochondrial genes (COI, COII and ND5). It is the case of host-specific seed predator, *Curculio hilgendorfi*, where sample size n=204 presented 114 haplotypes (2709bp) and $h$= 0.969, $\pi$= 0.006 (Aoki and Murakami, 2008); and host-specific leafminer, *Rhynchaenus dorsoplanatus*, n=171 show 90 haplotypes (2343bp) with $h$=0.973 and $\pi$=0.001. And the generalist bark beetle, *D. approximatus*, that analyses cyt-b (492bp) from n=71 and show 29 haplotypes (Sánchez-Sánchez, et al. 2012)

In conclusion, using COI gene as a unique genetic marker, in *T. compacta*, we did not find support for the hypothesis of higher genetic diversity on generalist over specialist insects**.**

**Host-plant specificity of *T. compacta***

Half of the most frequent haplotypes from *T. compacta* were found on different *Datura* host-plant species and some haplotypes with low frequency were restricted to a host-plant species (Fig. 3). The same case occurs in *C. sikkimensis* where the most frequent haplotypes were found in three out of four host-plants, and others of lower frequency were present in one host-plant (Aoki, *et al.,* 2009). Haplotype association to a host-plant has been found in *Anthonomous grandis* too, among wild and commercial cotton (Barr, *et al.,* 2013). Here the haplotypes networks are an important resource that permits mapping the distribution over the host-plant distribution, and at the same time infer evolutionary relationships of the insects collected in different host-plant. Nevertheless, the historicity of the association is unknown and the use of several other genetic markers could give us a better idea of the influence of host-plant on genetic variation.

Using only COI, we did no find genetic structure associated to a *Datura* host species in *T. compacta* (Fig. 2: Supplementary Material). In contrast to others generalist weevils like *A. grandis* where four nuclear genes additional to mitochondrial markers, show some degree of population differentiation among host-plants (Kuester, *et al.,* 2012).

An important remark of the speciation process in insects is that host plant can be only one source of selection, and expected genetic variation expected might depend on several factors, such as few or many loci responding to this selection pressure, the hitchhiking and the selective sweeps and of course, others sources of selection (e.g. Supple *et al.,* 2014).

**Phylogeographic pattern of generalist and specialist weevils**

The geological history of North America begins at the late Cretaceous 100-45 m.a. with the formation of Sierra Madre Oriental (SMOr), followed by the recurrent marine introgression at the Central Plateau of Mexico until the rise of Sierra Madre Occidental (SMOc) 30-28 m.a, The Transmexican Volcanic Belt (TVB) is more recent with its last episodes of volcanism during the Pliocene and the Quaternary. These in combination with interglacial cycles and climate fluctuations (Morán-Zenteno, 1984) left an impact on the biota of these areas. Which means that the geological history may have had a greater impact on *T. soror* than on *T. compacta*, like other weevils distributed on the highlands of Mexico (Sánchez-Sánchez *et al.*, 2012; Anducho-Reyes *et al.*, 2008). Among these "highland weevils", in fact, there is population structure associated to the main mountain systems: SMOr, SMOc and TVB (De-la-Mora *et al.*, 2015; Sánchez-Sánchez *et al.*, 2010; Anducho-Reyes *et al.*, 2008).

The history of the North American warm deserts biota has revealed vicariate events among taxa distributed in this area (e.g. Leache & Mulcahy, 2007; Bryson, et al., 2012; Mantooth, *et al.*, 2013), barriers to gene flow identified in this biota are Central Valley, Colorado River and floristic provinces. These barriers, particularly Colorado River, seem to have slightly influence in the population structure of *Trichobaris compacta*, we fond there are more genetic diversity at the north of its distribution still they were sampled in the same host plant (*D. wrightii*), than in the populations sampled in central Mexico where they were found in several *Datura* species.

It is possible that *T. compacta* recently has dispersed to *Datura* host species until attaining its actual distribution range. These could be in favor of the hypothesis of neartic origin of this weevil species as in *Sphenophorus*, *Smicronyx*, *Ophyrastes*, some *Curculio*, *Apleurus*, *Cleonidious* and certain *Listronotu*s, where their recent origin and their little diversification has resulted in few Mexican endemics (Anderson & O'brien, 1996),

**CONCLUSIONS**

We did not find support for the hypothesis of higher genetic variation in generalist vs. specialist herbivores, when compared *T. compacta* and *T. soror*. Some haplotypes were restricted to a host-plant species, but in general no population structure was associated to a particular *Datura* species. The phylogeographic and phylogenetic patterns show that *T. compacta* is a recent species with populations slightly structured over its current distribution range.

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### Compliance with ethical standars

**Conflict of interest**. Authors do not have any financial relationship with the organization that funded the research. The authors declarate they have no conflict of interest.

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**Supplementary Material-**Population Structure Analisis

Figure 1S.Bayesian clustering of population structure for *Trichobaris compacta* using COI gene (663bp), K=2.

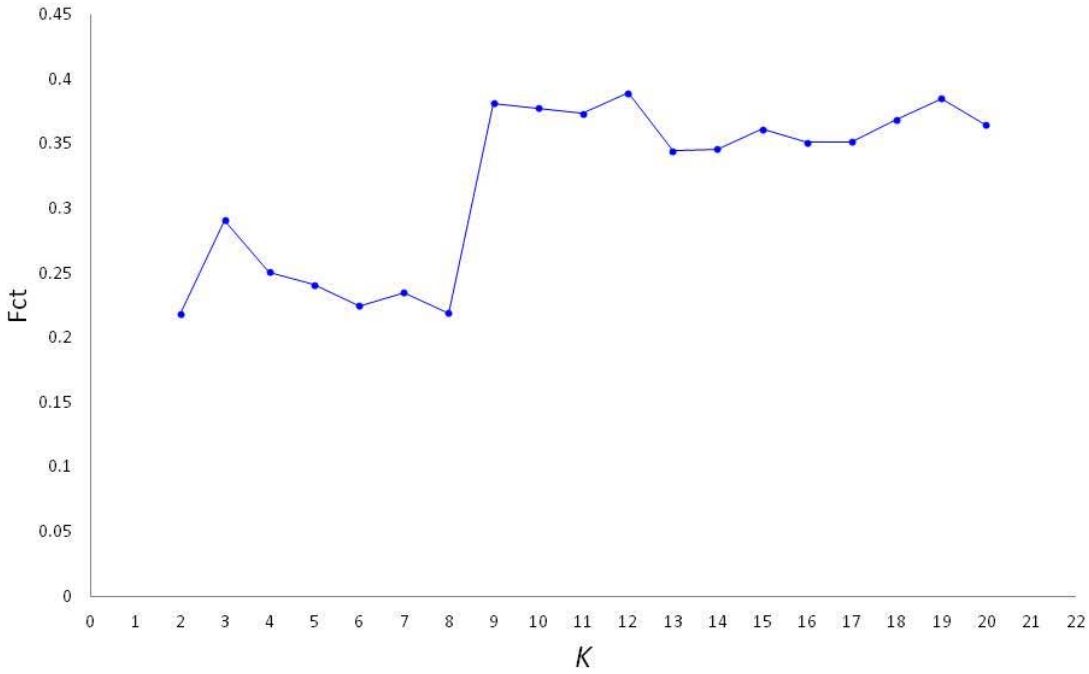


Figure 3S. Results from SAMOVA analysis of *Trichobaris compacta* populations, using COI gene (663bp).Where *K*=3 is group 1 (0Az,10SD, 11SD, 12SD, 13SD, 15SD,1Az, 20SD, 2Az, 2SD, 314, 3Az, 405, 4Az, 516, 5SD, 6Az, 8SD, AP, OG, 129) group 2 (19SD, 3SD) and group 3 (14SD).

**Artículo de investigación 3.**

# Evolution of the genus *Trichobaris* Le Conte (Coleoptera: Curculionidae): parasite weevils of *Datura,* Potato, Tomato and Tobacco.

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## Abstract

Diversification of weevils is tightly linked to the evolution of flowering plants. Yet, to fully understand their diversification, the analysis of it at lower taxonomic levels and their relationships with their host plants are badly needed. The genus *Trichobaris* comprises 8-13 species described in the literature, that parasite either the fruits of plants in the genus *Datura,* or the stem of various wild and cultivated *Solanaceae*, such as potato, tobacco and tomato plants. The aim of this study is to obtain a phylogenetic hypothesis for the genus *Trichobaris*, based on four markers: 18S and 28S nuclear ribosomal DNA and two mitochondrial genes 16S and COI genes. Also, we also conducted a morphogeometric analysis of the species in order to discriminate species morphologically. A total of 75 landmarks, including the shape of the *rostrum*, the *pronotum* and body shape, were obtained for each specimen. Haplotype networks for each species were built, using variation in the COI gene, and the host plant at in which they were collected was mapped. Finally, reconstruction analysis of the ancestral host plant was estimated with maximum likelihood in order to identify those host plants that could represent recent colonization events. Our results show convergences in morphology and different morphotypes in the phylogeny. The estimation of the ancestral host plant of *Trichobaris* indicates historical associations mainly with *Datura stramonium* and *D. wrightii,* and recent colonization to other *Datura* species.

**Resumen**

La evolución de los curculiónidos está íntimamente ligada a la evolución de las plantas con flor. Sin embargo para entender el proceso de diversificación de los insectos herbívoros, es necesario su análisis a niveles taxonómicos menores y su interacción con las plantas huésped. El género *Trichobaris* consta de 8 a 13 especies descritas en la literatura, que parasitan los frutos de las plantas del género *Datura*, así como el tallo de varias solanáceas silvestres y cultivadas, como la papa, tabaco y tomate. El objetivo de este estudio fue estimar la filogenia molecular del género *Trichobaris*, basada en dos marcadores nucleares (18S y 28S) y dos mitocondriales (16S y COI). A la vez, se realizo un análisis morfogeométrico para delimitar morfológicamente las especies; se obtuvieron un de 75 *landmarks* para cada espécimen, incluyendo la forma del *rostrum*, del *pronotum* y el cuerpo. Se obtuvo una red de haplotipos para cada especie usando la variación en el gen COI, y se mapeó las especies de planta huésped donde fueron colectados. Finalmente, se estimo la planta huésped ancestral y se determinaron los casos en los que las plantas huésped constituyen eventos recientes de colonización. Nuestros resultados muestran convergencias en la morfología y distintos morfotipos en la filogenia. La reconstrucción de la planta huésped ancestral indica asociaciones históricas principalmente con *Datura stramonium* y *D. wrightii*, y colonizaciones recientes entre las demás especies de *Datura*.

**Keywords:** *Datura* colonization to host-plant ancestral host-plant *Trichobaris* Insect-plant interaction

## Introduction

The speciation proccess is seldon seen in nature. The comparison of populations with different divegerence levels provides insights on how speciation proceeds (Nosil, 2012; Supple *et al.*, 2014). The phylogenetic approach provides a hypothesis of relationships among species based on morphological and/or molecular data providing an indirect record of the sequence of lineage-divergence events that have led to extant species (Barraclough & Nee, 2001). Ideally one should sample all the species of a taxonomic group, such as a genus, ensuring these species reflect evolutionary entities within the clade, and rule out the effects of other processes as well explanations for observed patterns (Avise & Jhons, 1999; Holt & Jonsson, 2014). The phylogeographic approach allows reconnaissance of geographic distribution and spatial genetic structure of populations within a species at early states of population differentiation, influenced either by drift and gene flow and/or

promoted by selection (Avise, 2000; Gavrilets, 2003). Both phylogenetic and phylogeographic approaches can help to further understand of evolution of a group of species.

Speciation in phytophagous insects has long been associated with plant diversification (Ehrlich & Raven, 1964; Farrell, 1998). Phytophagous insects in the order Coleoptera include the superfamilies Chysomeloidea and Curculionoidea (Grimaldi and Engel, 2005). Curculionidae beetles, known as weevils, include *ca*. 62 000 described species (Oberprieler *et al.,* 2007). Presumably such a diversity of phytophagous insects could be associated mainly with the diversity of angiosperms (McKenna, et al. 2009) and the ability of them to exploit different plant tissues (Marvaldi *et al.,* 2002). But its diversity is also associated to presumed key innovations, such as the length and shape of rostrum, endophagous larvae and geniculate antennae, and in some groups also to association with fungi (Oberprieler *et al*., 2007; Matsubayashi *et. al.,* 2009; e.g. Suppler *et al.,* 2014).

Weevils are pests of many economically important plants such as chestnut (Avtzis *et al.,* 2013; Kuroki and Kodama, 1987), sugar (Lemic *et. al.,* 2016), banana (Shankar *et al*, 2015), cotton (Barr el. al. 2013), raspberry (Parra et al. 2009) avocado (Castañeda-Vildozola *et. al.,* 2015; Bierig, 1939), mango (Basio, 1994), pecan (Mynhardt, 2006), pepper (Capinera, 2005), alfalfa (Iwase *et al.,* 2015) and sunflower (Charlet, 1983). At the macroevolutionary level, the historical relationship between these weevils and their host plants has been rarely explored. Also, discerning between ancestral and recently colonized host plant species is a poorly tackled topic (e.g. Iwase *et al.,* 2015; Kuester *et al.,* 2012). Some studies have shown the relevance of this the ecological and evolutionary consequences of host shifts on biological control and/or to sustain productivity (Olivieri *et al.,* 2008).

At the microevolutionary level, the effect of the host plant on the genetic variation of weevil species in a genus is a poorly explored question (e.g. Hernández *et al.,* 2010; Kohyama *et al.,* 2014). In some cases, a clear relationship between the genetic variation of weevils and their host plants has not been documented. For instance, in the stenophagous capitulum weevil (*Larinus cynarae*) the primary distribution of their genetic diversity indicates geographic division followed by branching of *L. cynarae* lineages into different host plants (Briese *et al.,* 1996). Howhever, in the boll weevil (*Anthonomus grandis*) three morphological forms have been described associated to species of *Gossypium,* but genetic differentiation is related to their geographical distribution (Kuester *et al.,*

2012), although, some haplotypes appear to be related to wild or cultivated cotton (Barr *et al.*, 2013). In an extreme case, the genetic variation in the alfalfa's weevil, *Hypera postica,* was not associated either to its host plant or to geographic distribution (Iwase *et al.,* 2015). However, coevolution between weevils and their host plants can result in a clear association of the genetic variation of both organisms e.g. *Curculio hilgendorfi* and *Castanopsis sieboldi* (Aoki *et al.,* 2011), or a shared phylogeographical pattern between them (Aoki *et al.,* 2009).

Weevils of the genus *Trichobaris* (Curculionidae: Baridinae) parasitize various species of plants in family Solanaceae, particularly, species of the genus *Datura*. Of the 13 *Trichobaris* species, six parasite *Datura*, three parasites the stalks of potato, tobacco and tomato (*Solanum tuberosum, Nicotiana attenuata* and *Physalis sp.),* and four parasite wild species of *Solanum* (*S. eleagnifolium, S. rostratum, S. carolinense*) (Barber, 1935). The life cycle of these weevils is closely associated to their host plants; for instance, the larva of *T. bridwelli* can not survive in a different host (Cuda, 1991). Since Barber's (1935) review, the genus has not been studied again in this depth. His work on *Trichobaris* included specimens from a wide range of locations and morphological descriptions in great detail. Nevertheless, Barber (1935) pointed out the issues that he could not be then resolved including the need of precise information about the host plant. And the relevance of body shape and size in delimiting morphological species. Thus, the study of speciation makes necessary to identify the species, their host plant and the determination of their evolutionary relationships.

In this study, we used geometric morphometric information and molecular variation, to analyze the *Trichobaris* species described by Barber (1935). Then, we assess the host distribution on the phylogeographic structure and mapping the ancestral host plant, to investigate the influence of the host plant on *Trichobaris* evolution. Thus, we cover here four important aspects related to speciation of weevils. First, using a morphogeometric approach we define the number of species in the genus; second, we estimate the phylogenetic relationships among species using the sequences of four genetic markers; third, we describe the distribution of genetic variation associated to the host plant species based on COI haplotypes network; and finally we dated the divergence events and estimated the ancestral host plant species of *Trichobaris* using the COI haplotype phylogeny.

## 2 Material and methods

### 2.1 Sampling and specimens examined

A total of 168 insects (including adult and larva stages) were collected in 33 localities across the *Trichobaris* distribution range in Mexico and the United States of America (Table 1). Almost all described species of *Trichobaris* Le Conte (Barber, 1935) were collected. Collectively, they were associated to eight different species of *Datura*, four wild species of Solanaceae (*Physallis sp*., *Solanum eleagnifolium, S. carolinense* and *S. rostratum*) and one cultivated species of Solanaceae (*S. tuberosum*). Additionaly, we examined and photographed 134 specimens from the following collections: CNIN (Universidad Nacional Autónoma de México), COLPOS (Colegio de Posgraduados, México) and MCZ (Harvard University).

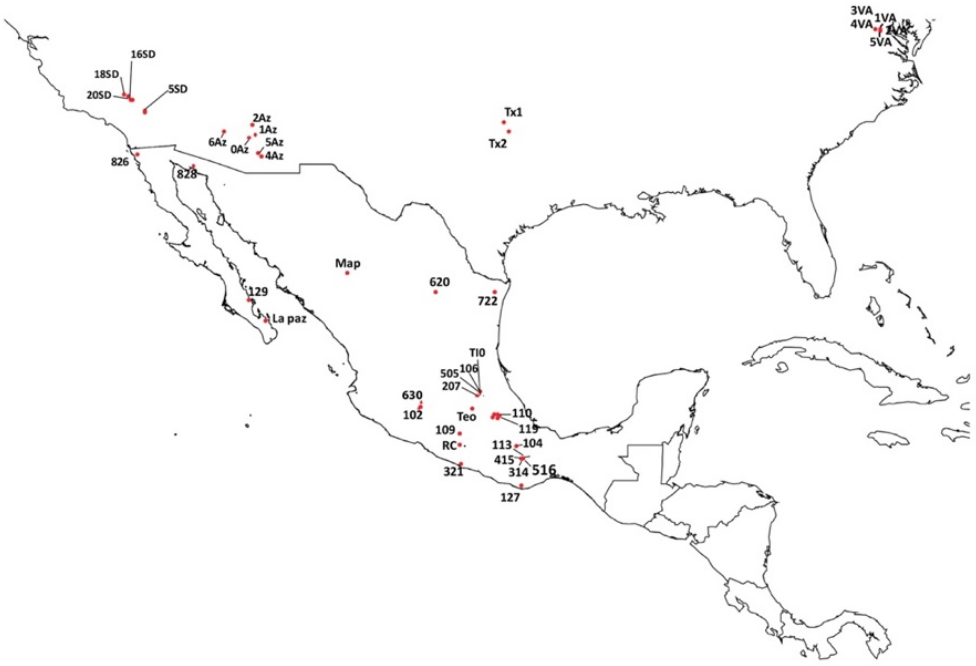



Figure 1. *Trichobaris* sampled localities in USA and Mexico. Labels represent sample sites as indicated in Table 1.

Table 1. Sampling sites of *Trichobaris* weevils on their host plant species in Mexico and USA.

| Number | Host plant | State | Country | Sampling site | Coordinates | # insects DNA data | # insects Morphology data |
|---|---|---|---|---|---|---|---|
| 1 | *D. discolor* | Baja California | Mexico | 129 | 26° 0' 21.46''N, 111° 20' 35.34''W | 5 | 7 |
| 2 | *D. inoxia* | Hidalgo | Mexico | 505 | 20° 34' 54.50''N, 99° 33' 50.58''W | 5 | 2 |
| 3 | *D. inoxia* | Oaxaca | Mexico | 516 | 16° 28' 46.10''N, 96° 13' 3.51''W | 1 | 5 |
| 4 | *D. pruinosa* | Oaxaca | Mexico | 415 | 16° 40' 2.60''N, 96° 22' 48.98''W | 1 | ____ |
| 5 | *D. quercifolia* | Hidalgo | Mexico | 207 | 20° 19' 3.79''N, 99° 9' 34.98''W | 20 | 12 |
| 6 | *D. stramonium* | Puebla | Mexico | 119 | 19° 5' 38.49''N, 98° 24' 34.89''W | 6 | ____ |
| 7 | *D. stramonium* | Oaxaca | Mexico | 104 | 17° 14' 11''N, 96° 25' 53.77''W | 9 | ____ |
| 8 | *D. stramonium* | Hidalgo | Mexico | 106 | 20° 8' 18.98''N, 98° 55' 21.38''W | 5 | ____ |
| 9 | *D. stramonium* | Puebla | Mexico | 110 | 18° 54'8.23''N, 98° 26' 21.15''W | 19 | ____ |
| 10 | *D. stramonium* | Puebla | Mexico | 109 | 18° 56' 27.15''N, 98° 6' 53.46''W | 18 | 24 |
| 11 | *D. stramonium* | Oaxaca | Mexico | 113 | 16° 55' 11.92''N, 96° 23' 6.10''W | 20 | 1 |
| 12 | *Physallis sp.* | Hidalgo | Mexico | T18 | 20° 35' 23.59'N, 99° 37' 11.22''W | 2 | 2 |
| 13 | *Solanum eleagnifolium* | Tamaulipas | Mexico | 722 | 25° 58' 45.05''N, 98° 5' 45.66''W | 1 | ____ |
| 14 | *Solanum rostratum* | SanLuisPotosí | Mexico | 620 | 23° 33' 36.99''N, 100° 37' 41.69''W | 8 | 1 |
| 15 | *D. wrightii* | California | USA | 5SD | 33°46'4.14"N, 116°19'28.23"W | 1 | 3 |
| 16 | *D. wrightii* | California | USA | 8SD | ____ | 3 | ____ |
| 17 | *D. wrightii* | California | USA | 20SD | 33°59'46.45"N, 116°34'43.69"W | 1 | ____ |
| 18 | *D. wrightii* | Arizona | USA | 0Az | 32°35'32.79"N, 110°50'56.54"W | 3 | 1 |
| 19 | *D. wrightii* | Arizona | USA | 1Az | 32°36'50.10"N, 110°49'57.91"W | 3 | ____ |
| 20 | *D. wrightii* | Arizona | USA | 2Az | 32°58'42.19"N, 110°46'8.08"W | 3 | 3 |
| 21 | *D. stramonium* | Arizona | USA | 4Az | 32° 3'48.57"N, 110°17'3.42"W | 1 | 1 |
| 22 | *S. eleagnifolium* | Arizona | USA | 5Az | 32° 1' 54.69''N, 110° 18' 40.51''W | 2 | ____ |
| 23 | *D. wrightii* | Arizona | USA | 6Az | 33° 5'25.83"N, 112° 2'1.26"W | 3 | 2 |
| 24 | *S. eleagnifolium* | Texas | USA | Tx1 | 33° 52' 55.49'' N, 98° 28' 53.04'' W | 2 | 1 |
| 25 | *S. eleagnifolium* | Texas | USA | Tx2 | 32° 54' 27.50''N, 97° 34' 50.64''W | 1 | ____ |
| 26 | *S. carolinense* | Virginia | USA | 4VA | 37°37'22.4"N, 77°59'13.1"W | 4 | ____ |
| 27 | *S. tuberosum* | Virginia | USA | 4VA | 37°37'11.4"N, 77°59'18.8"W | 2 | ____ |
| 28 | *D. stramonium* | Virginia | USA | 5VA | 37°30'38.5''N, 77°42'12.9''W | 2 | 4 |
| 29 | *S. carolinense* | Virginia | USA | 1VA | 37°39'49.8''N, 77°53'30.1''W | 1 | ____ |
| 30 | *S. carolinense* | Virginia | USA | 2VA | 37°32'28.6''N,77°53'36.8''W | 4 | ____ |
| 31 | *S. carolinense* | Virginia | USA | 3VA | 37°32'56.3''N,77°55'01.8''W | 5 | ____ |
| 32 | *D. stramonium* | Virginia | USA | 3VA | 37°31'31.5''N,77°52'11.5''W | 2 | ____ |
| 33 | *D. ceratocaula* | Durango | Mexico | Map | 25° 50' 1.35''N, 103° 50' 55.46''W | ____ | 5 |
| 34 | *D. stramonium* | Michoacán | Mexico | 102 | 19° 40'46.19''N, 101° 15' 12.68''W | ____ | 3 |
| 35 | *Datura sp.* | Baja California | Mexico | Uru | 31°34'03.2''N,116°25'19.6''W | ____ | 6 |
| 36 | *Datura sp.* | Baja California | Mexico | SAN | 32°06'55.8''N,166°30'02.8''W | ____ | 7 |
| 37 | *Solanum sp.* | Michoacán | Mexico | 630 | 19° 41' 9.87''N, 101° 13' 46.56''W | ____ | 3 |
| 38 | *D.quercifolia* | Guanajuato | Mexico | 230 | 20°25'20.6''N,99°58'37.9''W | ____ | 18 |
| 39 | *D. stramonium* | Edo. de Mexico | Mexico | Teo | 19° 40' 48.75''N, 98° 52' 26.51''W | * | 2 |
| 40 | *D. stramonium* | Guerrero | Mexico | RC | 17° 32' 27.34 N,99° 28' 19.27''W | * | 1 |
| 41 | *D. discolor* | Oaxaca | Mexico | 314 | 16° 47' 11.61''N, 96° 12' 42.34''W | * | 14 |
| 42 | *D. discolor* | BajaCaliforniaN. | Mexico | 828 | 32° 11' 28.14''N, 114° 55' 19.63''W | * | 7 |
| 43 | *D. wrightii* | California | USA | 16SD | 34°11'19.90"N, 116°26'4.89"W | * | 2 |
| 44 | *D. wrightii* | California | USA | 18SD | 34°14'9.57"N, 116°26'23.43"W | * | 2 |
| COLPOS | ____ | ____ | Mexico | ____ | ____ | ____ | 3 |
| IB,UNAM | ____ | BajaCaliforniaSur | Mexico | | 24 8' 33.51''N, 110 18' 45.91''W | ____ | 24 |
| MCZ, HU | ____ | ____ | ____ | ____ | ____ | | 76 |
| | | | | | Total | 163 | 242 |

(*) COI gene sequence from these populations is previously in De-la-Mora *et al.*, 2015; in press.

Because immature stages cannot be used to identify species, not all insects were used for analysis. Instead, larvae were used only when obtained from localities where no adults were found. Due to their small size non-destructive DNA extraction was possible so we decided to choose subsets of insects randomly selected for morphology and genetic analysis. The morphological analysis was based on a total of 245 insects (Table 1). The phylogenetic analysis was based in 158 insects, for which information about geographical distribution and plant host were documented. For phylogeographic analyses we included 189 COI sequences together with sequences from to two previous studies (*T. soror*, De-la-Mora *et al.,* 2015; and *T. compacta*, De-la-Mora *et al.,* in press). A total of 844 COI sequences from species of *Trichobaris* were analyzed (Table 1 and Fig. 1) which resulted on 198 haplotypes used to build a calibrated phylogeny and to map the ancestral host plant.

## 2.2 Geometric morphometrics

A total of 245 insects were identified (Barber, 1935) and photographed in frontal, lateral and dorsal views. All the images were identically oriented so the frontal view was positioned with the head at the middle of the photograph; the lateral view always was showed the left side, and the *mesopleuron* was placed in the middle; and in the dorsal view the *scutellum* was oriented towards the bottom and the head towards the top. Two different imaging methods were used: with a SteREO Discovery.V8 Zeiss stereomicroscope combined with AxioVision software for image processing (Instituto de Ecología, UNAM); and with a Canon EOS 6D camara mounted on StackShot automated macro rail ® Cognisys Inc. combined with Helicon Focus image processing software (Museum of Comparative Zoology, Harvard University).

Landmark analysis. For each specimen we assessed body shape with a total of 79 landmarks: 8 in frontal, 39 in lateral and 28 in dorsal views (Figure 2). They were quantified from a set of two dimensional coordinates with tpsDIG 1.4 software (Rohlf, 2004). To remove the differences due to scale, orientation and location from the landmark configurations, we used a minimum procrustes distance criterion, as implemented in MorphoJ (Klingenberg, 2011) to produce a set of partial Procrustes superimpositions of specimens. The coordinates obtained were transformed into relative warp scores to produce a W matrix. Shape variation among morphotypes was analyzed with a

Principal Component Analysis (PCA). To maximized individual differences according to species, we performed a Canonical Variate Analysis followed by a Discriminant Analysis among pairs of species. Both analysis where implemented in MorphoJ (Klingenberg, 2011). Once the phylogeny of *Trichobaris* was obtained (see below), we used pictures of body shape, *pronotum* and *rostrum* to evaluate a morphometric signal in the phylogeny (Klingenberg, 2011).

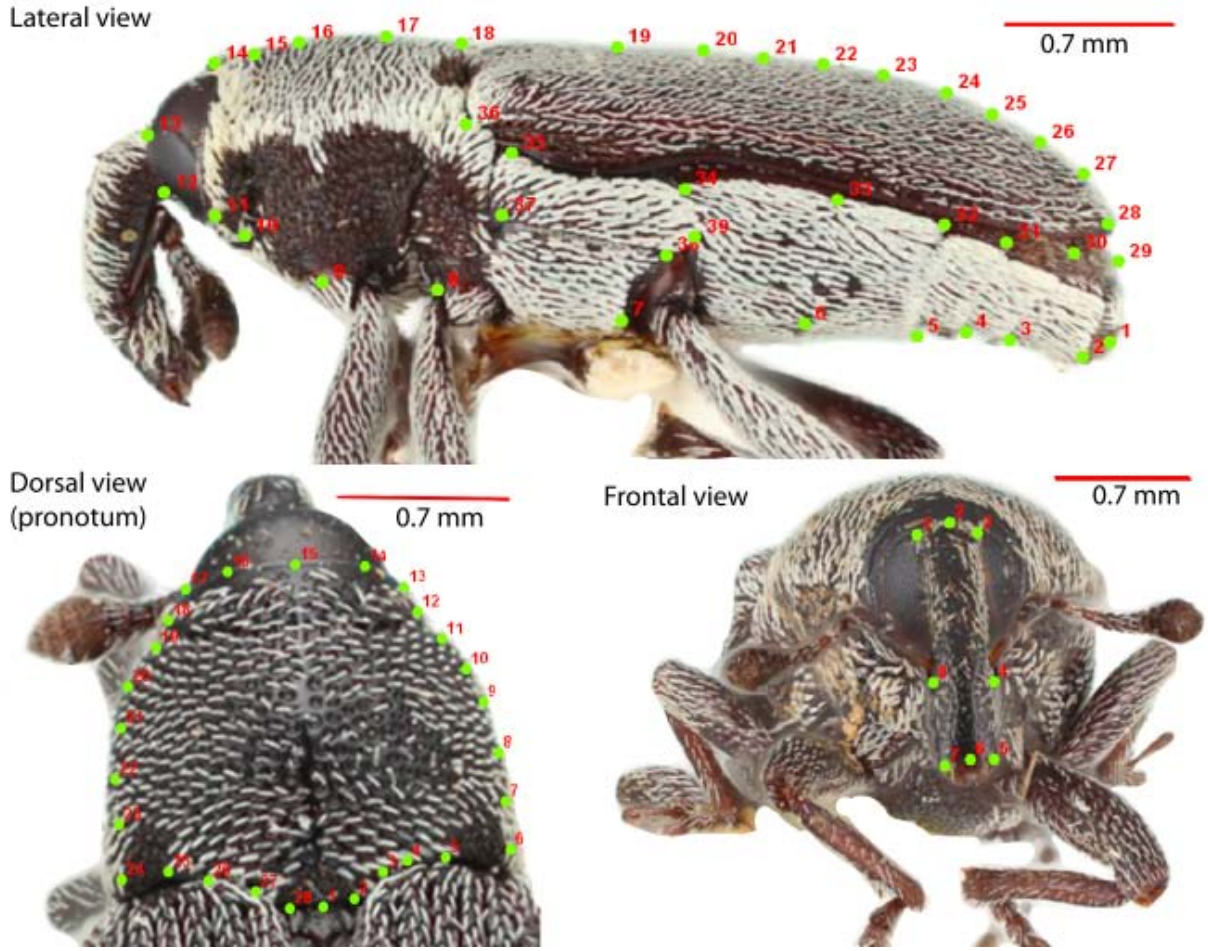



Figure 2. Photographs taken from a specimen showing the landmarks (green dots).

**2.3 DNA extraction, amplification and sequencing**.

Genomic DNA was extracted from insects' whole body using the DNeasy Blood & Tissue kit (Qiagen). The genetic markers used to build the *Trichobaris* phylogeny were four: two

mitochondrial genes (CO1 and 16S) and two nuclear genes (18S and 28S). The primers used were COA3107 and COS2183N (Sota, 2004), LR-J-12887 F and LR-N-13398R, 28S and 28SR, Insect18S-MIDF and Insect18S-MIDR (from Ikeda et al., 2008) for COI, 16S, 28S and 18S respectively. All PCR reactions were performed in volumes of 19.8µl: buffer 10x 2µl, $MgCl_2$ 20x 1µl, DNTPs 10mM 0.8µl, primer forward 10mM 1µl, primer reverse 10mM 1µl, Taq Amplificasa® 1µl, DNA 50mM 1µl and $H_2O$ 12µl. The amplification conditions were the same for all markers: an initial period of 5 min at 95 ° C followed by 35 cycles of 1min at 94 ° C, 55 ° C 1.2min and 1min at 72 ° C, with a final extension of 7 min at 72 ° C, with the exception of the 16S marker whose annealing temperature was 50 ° C (Sota, 2004). Samples were sent to sequence at the University of Washington's High-Throughput Genomics Center. In ABI 3730XL sequencer and the ABI Prism kit Cycle Sequencing Big Dye Terminator Ready Reaction (Applied Biosystems).

Sequences processings was done with the Sequencher® 5.0 software (Gene Codes Corporation). The alignment was carried out by the FFT-NS-1 progressive alignment method, using the online version of the software MAFFT v7 (http://mafft.cbrc.jp/alignment/server/).

**2.4 Estimation of genetic variability and phylogenetic analysis**.

The phylogeny was based on 156 specimens. The amount of genetic variability in COI, 16S, 18S and 28S was estimated with the software DNAsp ver. 5.1 (Rozas 2003). The total number of haplotypes, mutations, segregating sites ($S$), nucleotide diversity ($\pi$), haplotype diversity ($h$) and theta ($\theta$) using the Nei's (1987) equations, were estimated for the mitochondrial sequences (COI, 16S) and for nuclear sequences (18S and 28S).

Phylogenetic analyses were conducted on each locus separately, and on the concatenated matrix *Sitophilus oryzae* (Curculionidae) was specified as outgroup. Phylogenies were estimated using Bayesian Inference with the BEAST software. v1.4.7. (Drummond & Rambaut 2007). The analysis were executed using 100 000 000 MCMC, 10000 "burn in" with the GTR + I molecular replacement model using a fixed substitution rate of 1 substitutions/site/unit time (default). The running parameters were checked in Tracer v. 1.6 (Rambaut *et al.*, 2014) and the trees were summarized with TreeAnnotator v 1.4.8 (Rambaut & Drummond 2008a). Finally, the phylogenetic tree was visualized in FigTree v 1.1.2 (Rambaut & Drummond 2008b).

## 2.5 COI haplotype network and host plant.

Using the gene variation at the COI gene sequence from two previous publications (De-la-Mora *et al.*, 2015 and De-la-Mora *et al.* in press) and the ones obtained in this study, we compared the measures of genetic diversity within and between species in DNAsp software (Rozas, 2003). The relationship between haplotypes and their host-plant distribution was explored with haplotype networks built in the Network ver. 4.6.1.1 software (Bandelt, 1999), through median joining algorithm.

## 2.6 COI haplotype phylogeny

Using the COI haplotypes of all *Trichobaris* species we built the haplotype tree with Bayesian Inference using BEAST software. ver. 1.4.7. (Drummond & Rambaut, 2007). Analysis were executed using 10 000 000 MCMC, 1000 "burn in" with the GTR + I molecular replacement model. The phylogeny was calibrated with a 2% divergence per million years previously reported for COI in coleopterans (Nakamine & Takeda, 2008). The running parameters were checked in Tracer v. 1.6 (Rambaut, et al. 2014) and the trees were summarized in TreeAnnotator v 1.4.8 (Rambaut & Drummond, 2008a). The phylogenetic tree was visualized in FigTree v1.1.2 (Rambaut & Drummond, 2008b).

## 2.7 Mapping ancestral host plant analysis

The gene CO1 has been effective to recognize the host association (Jurado-Rivera, 2009). Here, we used the phylogenetic tree built from COI haplotypes instead of the species phylogeny, because the former has greater resolution that allows to relate terminals to a host-plant. Ambiguity was not taken into account to perform the analysis because the haplotypes present in two o more host plants were coded as being present only in the host plant where each is more abundant. Ancestral host utilization was estimated with Maximum Likelihood using the phytools package (Revell, 2012) implemented in R (R Development Core Team 2011). The eleven host plants collectively occupied by Trichobaris species were considered as a discrete character. The host plant of the outgroups was included in the analysis, but are biologically meaningless. considered for running the analysis although they lack of biological meaning. We used a continuous-time Markov chain model (Mk model) (Suchard *et al*., 2001) for trait evolution, which assumes that all transitions in character states have the same probability. To compare the ancestral host plant estimation, we performed two additional analyses

with parsimony and maximum likelihood using Mesquite ver. 3.0.4 (Maddison& Maddison, 2015). Because Mesquite allows a maximum of 10 states, we coded only ten states leaving one host as missing state as in the case of outgroups. Because we used a phylogeny of haplotypes and not of species, we did not attempt to estimate the phylogenetic signal. The theoretical foundation of phylogenetic signal is that individuals of the same species are more similar to each other in a given trait than to individuals of other species, while geographic variation of that trait among species is not taken into account. For ancestral reconstruction of the host plant, the geographic distribution of species in different hosts also is taken into account through the phylogeny of haplotypes. We did not test different models of reconstruction hypotheses because there is no empirical evidence or theoretical basis to suppose differential transition rates among states (host plants), for instance that some species of insects follow second chemistry compounds present in one species of plant and not in other. In addition, it is methodologically complex to analyze 12 discrete states because of the large number of parameters involved.

## 3 Results

### 3.1 Species identification and geometric morphometrics

From sampled insects and according to the Barber's key, we identified: *T. soror, T. major, T. compacta, T. mucorea, T. mucorea var. striatula, T. texana, T. cylindrica* and *T. trinotata.* The specimens from collections previously labeled were: *T. mucorea, T. trinotata, T. texana, T. championi* and *T. insolita.* A total of ten *Trichobaris* species were included in the geometric morphometric analysis, with eight species being clearly distinguishable: *T. soror, T. major, T. mucorea, T. mucorea var. striatula, T. texana, T. cylindrica, T. trinotata* and *T. compacta* (Fig.3; Fig. 1S Supplementary Material). Missing *T. pueblana T. compacta var. retrusa* and *T. bridwelly* from Barber descriptions. Among the three analyzed measurements, only the rostrum showed phylogenetic signal (Fig. 2S Supplementary Material). We found sexual dimorphism in this trait as is showed on the Figure 4. *T. soror, T. major* and *T. trinotata* have females with rostrum longer and thinner among the other females of *Trichobaris* species.

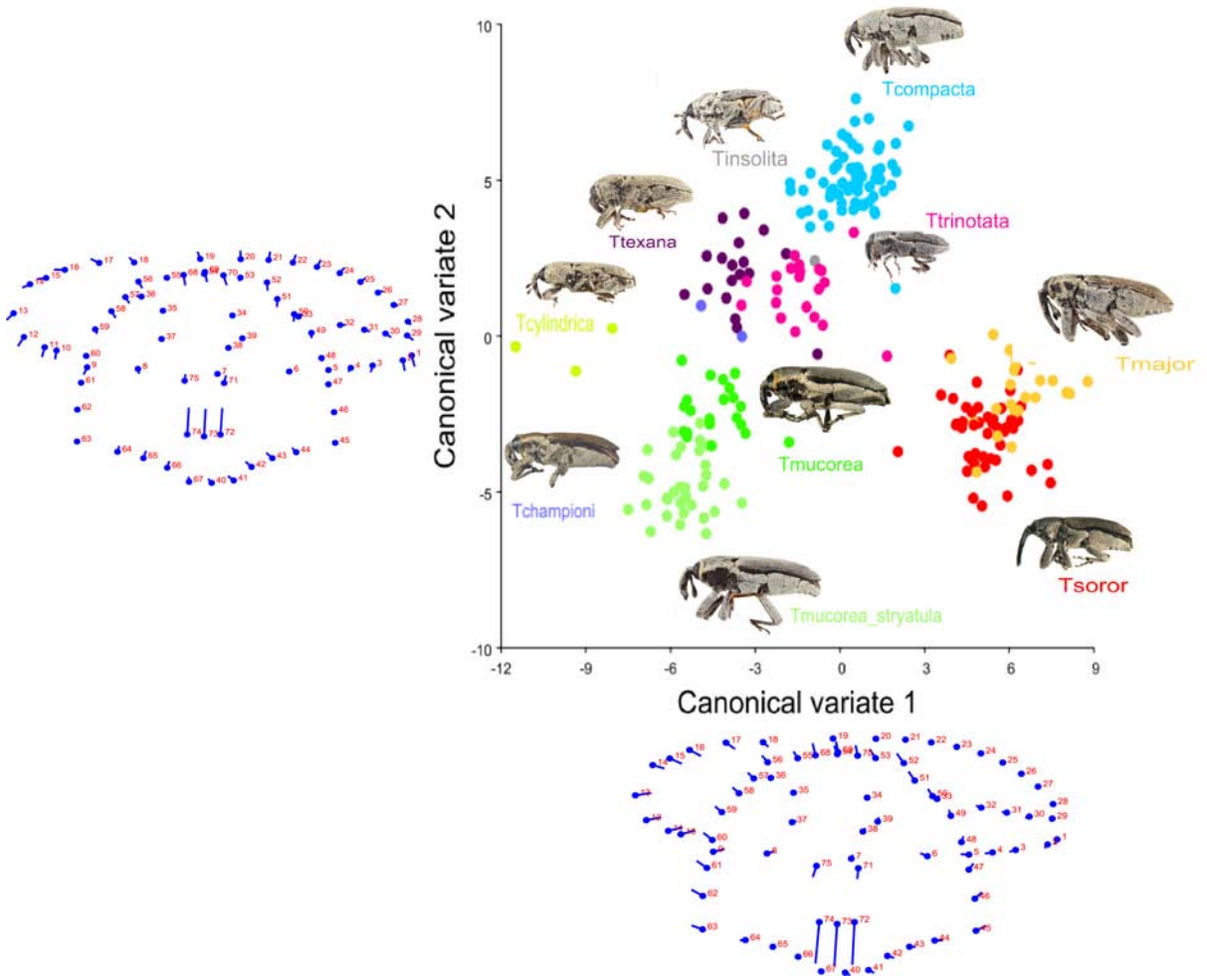
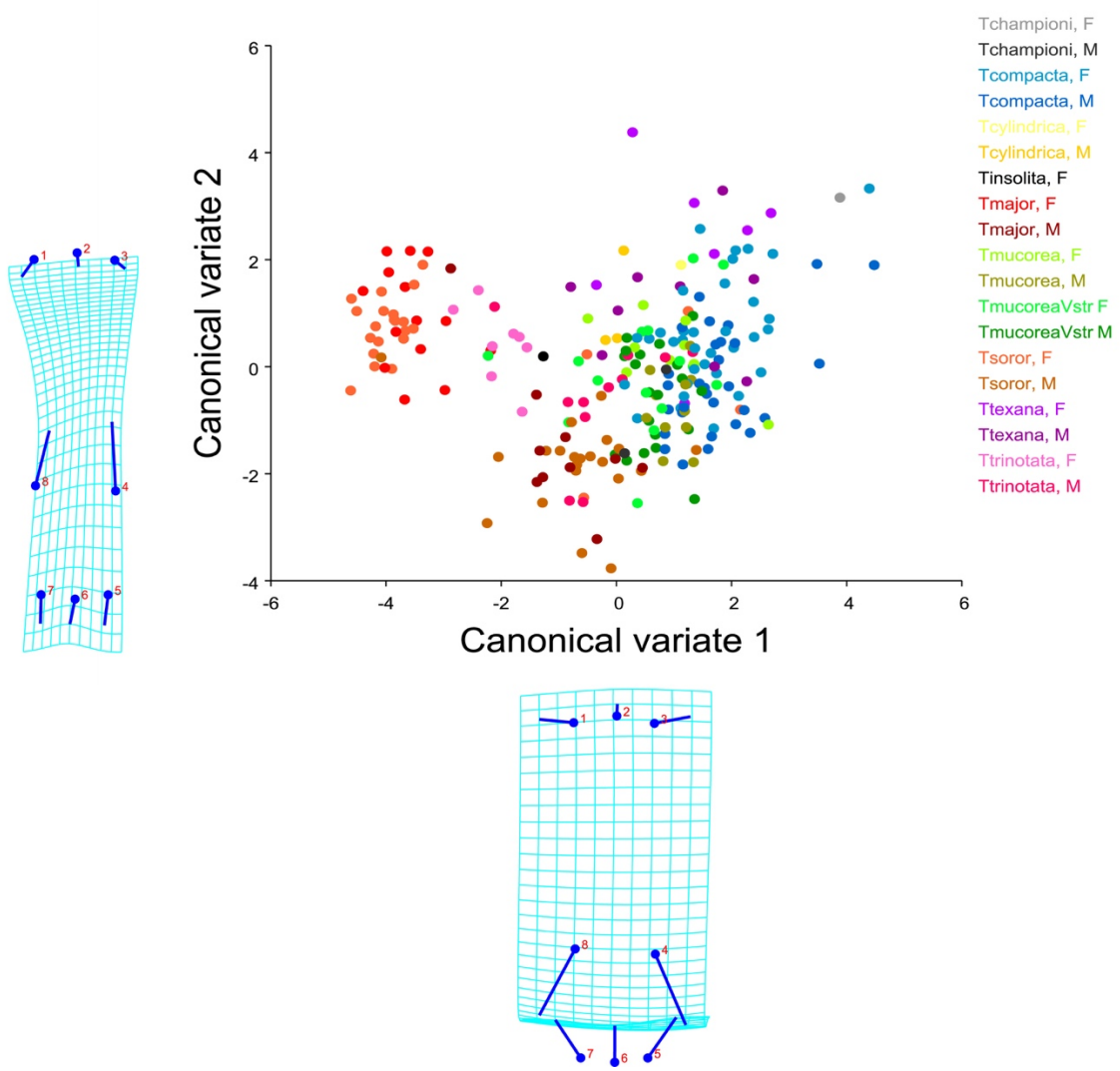



Figure 3. Analysis of Canonical Variance of the genus *Trichobaris*. Obtained by 75 landmarks from three photographs per specimen. Each individual is represented by a dot, colored according to the species to which it belongs. The photograph is representative of each species. The axes are the canonical variate 1 and 2 that represent the changes in shape.

### 3.2 Estimation of genetic variability and phylogenetic relationships

We sequenced 663bp of COI, 454bp of 16S, 429bp of 18S and 771 bp for 28S from 156 randomly selected insects. There was substantial variation in resolution among the four gene regions. For COI we found 59 haplotypes, with $h$=0.874, $\pi$= 0.052, $\theta$=0.064 and $S$= 26 For 16S, 26 haplotypes, with $h$=0.762, $\pi$= 0.025, $\theta$=0.06 and $S$=6. For nuclear sequences we found 18S, 15 consensus sequences with $h$=0.593, $\pi$= 0.005, $\theta$=0.020 and $S$=3. For 28S, 18 consensus sequences with $h$=0.518, $\pi$= 0.025, $\theta$=0.062 and $S$=10.

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286 Figure 4. Analysis of Canonical Variance of the *rostrum* of the species of genus *Trichobaris*.
287 Obtained by 8 landmarks per specimen. Males (M) and females (F) of each species are represented
288 by the same color darkest and lightest, respectively. The axes are the canonical variate 1 and 2 that
289 represent the changes in shape represented on the transformation gif outside of the graphic.

290

291 The Bayesian phylogeny provides an estimate of evolutionary relationships among *Trichobaris*
292 species (Fig. 5). Six species agree with morphological groupings and species reported. *Trichobaris*
293 *major* and *T. soror* belong to the same morphological group and are genetically indistinguishable.

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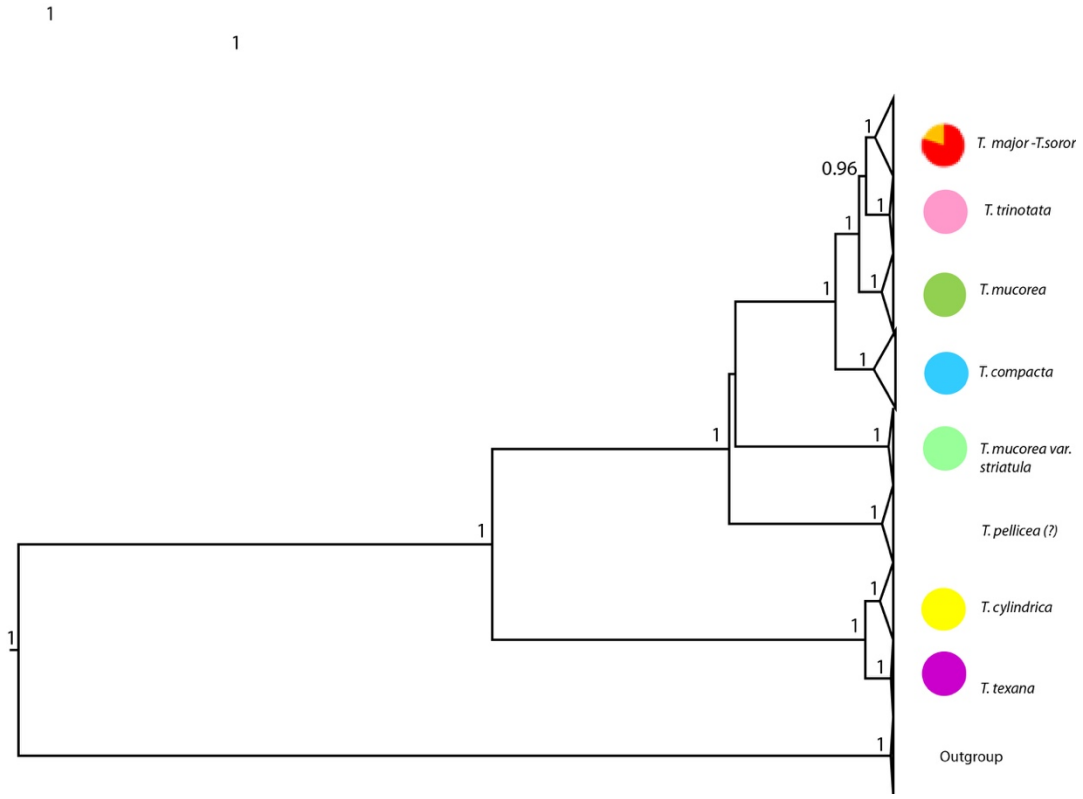


Figure 5. Bayesian phylogeny of *Trichobaris*. Phylogeny of *Trichobaris*, estimated with Bayesian Inference using 18S and 28S nrDNA and the mitochondrial 16S and COI. Colored circles correspond to the morphological differentiation of canonical variance analysis. (?) indicates that the species identity could not be corroborated by morphology.

Because larvae were used for this analysis, identification of some species required further confirmation, namely, *T. pellicea* was identified on the basis of its distribution and host plant. In general, the phylogenetic tree shows high posterior probability support for eight *Trichobaris* species.

### 3.3 Phylogeographic analysis

We obtained the eight haplotype networks of 189 haplotypes (Fig. 6) using only the variation of COI gene sequence (Table 2) for 844 insects, including the samples used for the estimation of the phylogeny (see above). The number of samples varied considerably among *Trichobaris* species, due

mainly to the differences on their distribution and abundance. Nevertheless, they provide the first insights about the spatial and temporal use of host plants in these weevil species.

The haplotype network of *T. mucorea* (n = 67) shows 16 haplotypes (Fig. 8S Supplementary Material). Most of them were sampled in *D. stramonium,* three were sampled in *D. inoxia* (Mu14, Mu15 and Mu16) and one (Mu12) was sampled on *D. pruinosa, D. discolor* and *D. stramonium.* Haplotype network of *T. soror* (n= 469) shows 99 haplotypes (Fig. 9S Supplementary Material). Most haplotypes were sampled in *D. stramonium* and 9 were sampled in *D. quercifolia.* Haplotypes So92, So93 and So94 were collected in *D. inoxia,* So93 also in *D. pruinosa* and So95 in *Solanum carolinense*. The haplotype network of *T. trinotata* (n= 21) shows four haplotypes mainly distributed on *S. carolinense* (Tr1, Tr3, Tr4), but also distributed on *S. tuberosum* (Tr2, Tr4) and *D. stramonium* (Tr4) (Fig. 10S Supplementary Material). The haplotype network of *T. compacta* (n=232) shows 49 haplotypes most of them were sampled in *D. wrightii,* 5 (Co42, Co43, Co44, Co45, Co46) on *D. reburra,* 8 (Co1, Co3, Co12, Co13, Co47, Co48, Co49) on *D. discolor*, 2 (Co1 and Co10) on *D. pruinosa* and 2 (Co1 and Co10) in *D. inoxia* (Fig. 11S Supplementary Material).

The haplotype network of *T. mucorea var. striatula* (n= 23) shows 13 haplotypes mainly distributed in *D. wrightii* and four of them in *D. discolor* (Ms5-Ms8) (Fig. 12S Supplementary Material). The mini haplotype networks of *T. texana* (n=7) shows three haplotypes distributed in *S. rostratum*, *T. cylindrica* (n=4) shows three haplotypes distributed in *S. eleagnifolium* and, finally, *T. pellicea* n=3 shows two haplotypes distributed in *D. stramonium* (Fig.13S Supplementary Material).

Table 2 Genetic diversity for *Trichobaris* species using 663bp of COI gene.

| Species | # insects | # haplotypes | *h* |
|---|---|---|---|
| *T. soror* | 469 | 99 | 0.785 |
| *T. trinotata* | 21 | 4 | 0.271 |
| *T. mucorea* | 67 | 16 | 0.701 |
| *T. compacta* | 232 | 49 | 0.709 |
| *T. mucorea var. striatula* | 23 | 13 | 0.932 |
| *T. texana* | 7 | 3 | 0.761 |
| *T. cylindrica* | 4 | 3 | 0.833 |
| *T. pellicea (?)* | 3 | 2 | 0.667 |
| **Total** | 826 | 189 | 0.905 |

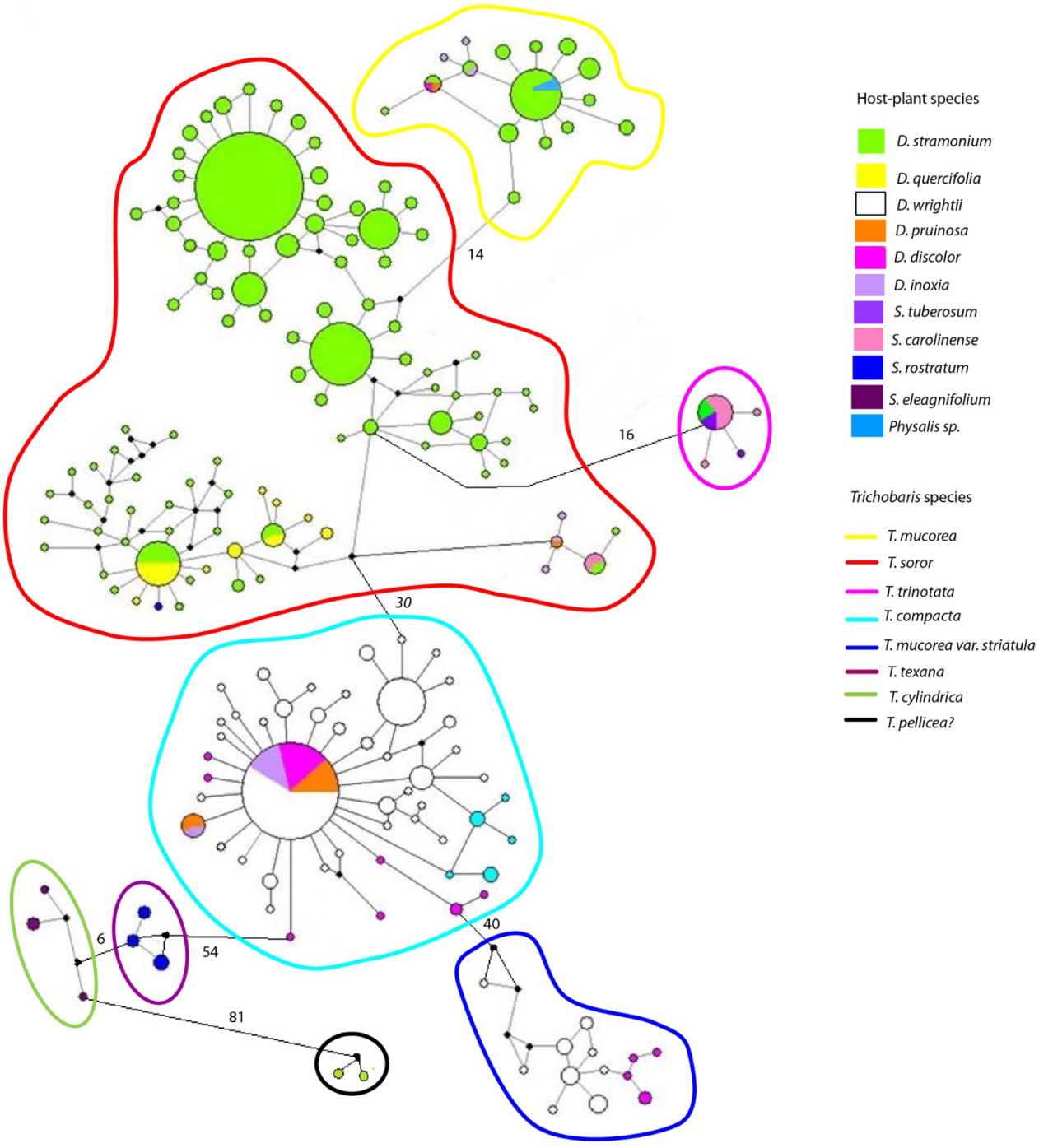


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Figure 6. Haplotype network of *Trichobaris* species estimated using the COI gene (663bp) using the *Median joining* algorithm. Black dots represent vectors and the numbers on branches indicate mutational steps (see Supplementary Material for a more detailed view: Figures S7-S13), (?) indicates that species identity could not be corroborated by morphology.

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**2.4 Haplotype calibrated phylogeny**.

Most of the clades inferred with high support based on the four loci (Fig. 3) were also inferred and strongly supported in the COI haplotype phylogeny (Fig. 7), only except for *T. pellicea* which appeared in a different position. The time-calibrated haplotype phylogeny shows that most of *Trichobaris* species are no older than 6.6 (±1.5) million years. The diversifications of *T. soror, T. trinotata, T. mucorea, T. compacta* and *T. mucorea* var. *striatula* haplotypes are very recent (less than 0.5 m.a.). The node number on Figure 7 compares age with trait state on Figure 8.

**2.4 Estimation of ancestral host plant species**.

We estimated the ancestral host plant on the COI haplotype phylogeny of *Trichobaris* species (Fig. 8). Here, we indicated the ancestral host in eleven nodes from (0-10) whose estimated likelihood values where not conclusive for nodes 0, 1, 2 and 4. Node 3 suggests with 0.528 of likelihood that *S. eleagnifolium* could be the ancestral host plant of *T. texana* and *T. cylindrica*. Likelihood values of 0.318 and 0.982 from nodes 5 and 7, respectively, support the hypothesis that *D. wrightii* was the ancestral host plant of *T. compacta* and *T. mucorea* var. *striatula*. And finally node 6, 8, 9 and 10, the probability that *D. stramonium* was the ancestral host plant for *T. soror, T. trinotata* and *T. mucorea* increases (0.272, 0.784, 0.862 and 0.983, at each node).

**4 Discussion**

This study constitutes the first integrative analysis (using morphological, phylogenic and phylogeographic approaches) of species of *Trichobaris* to investigate their association with plants of Solanaceae. Our results provide relevant insights to understand the evolution of *Trichobaris* in relationship with its plant hosts.

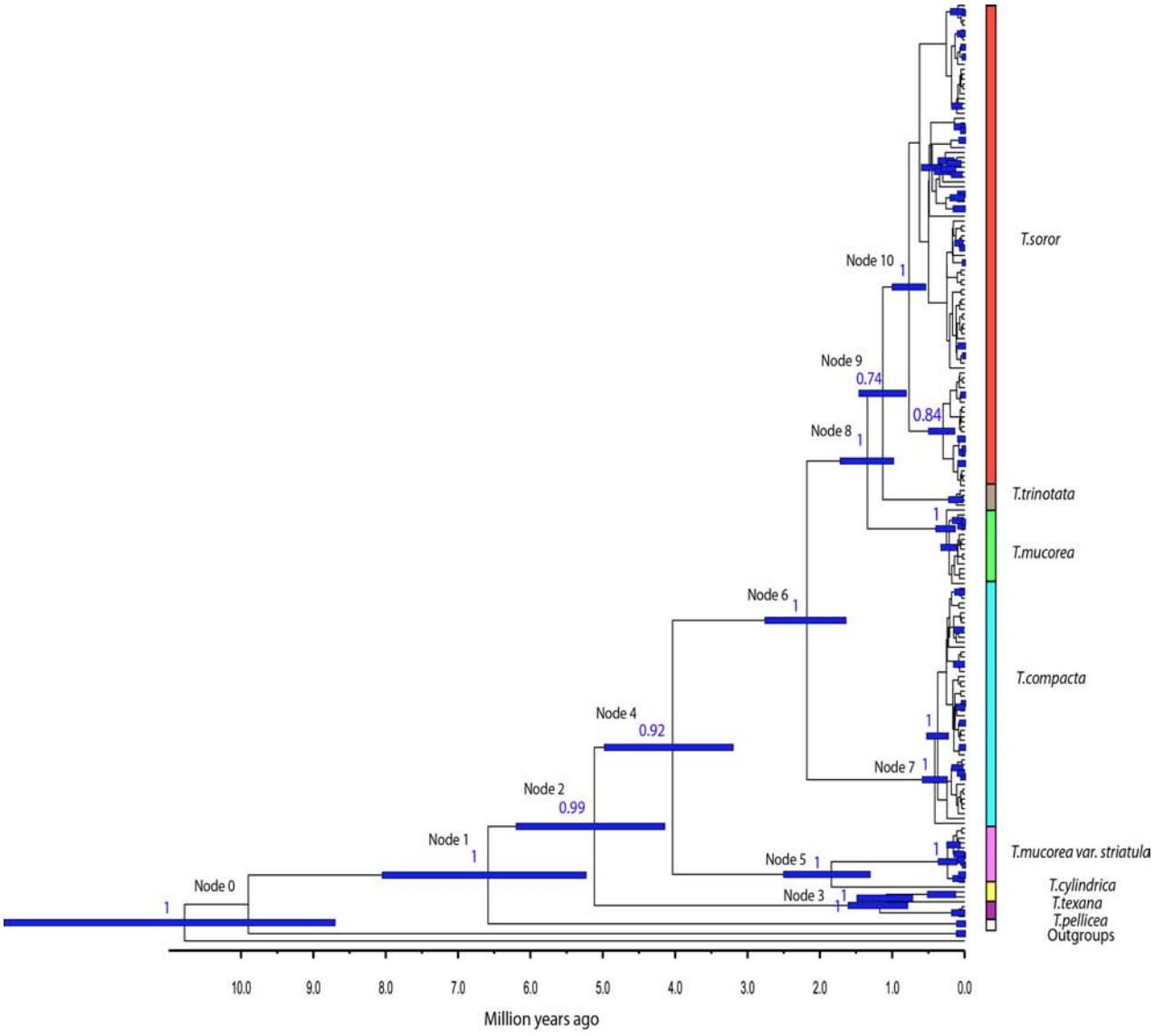


Figure 7. Calibrated COI haplotype tree of *Trichobaris* species. Node bars represent the variation on age estimation. Blue numbers on branches indicate node support (posterior probability). Node numbers are indicated for comparisons with estimation of ancestral host plant (see text for more information).

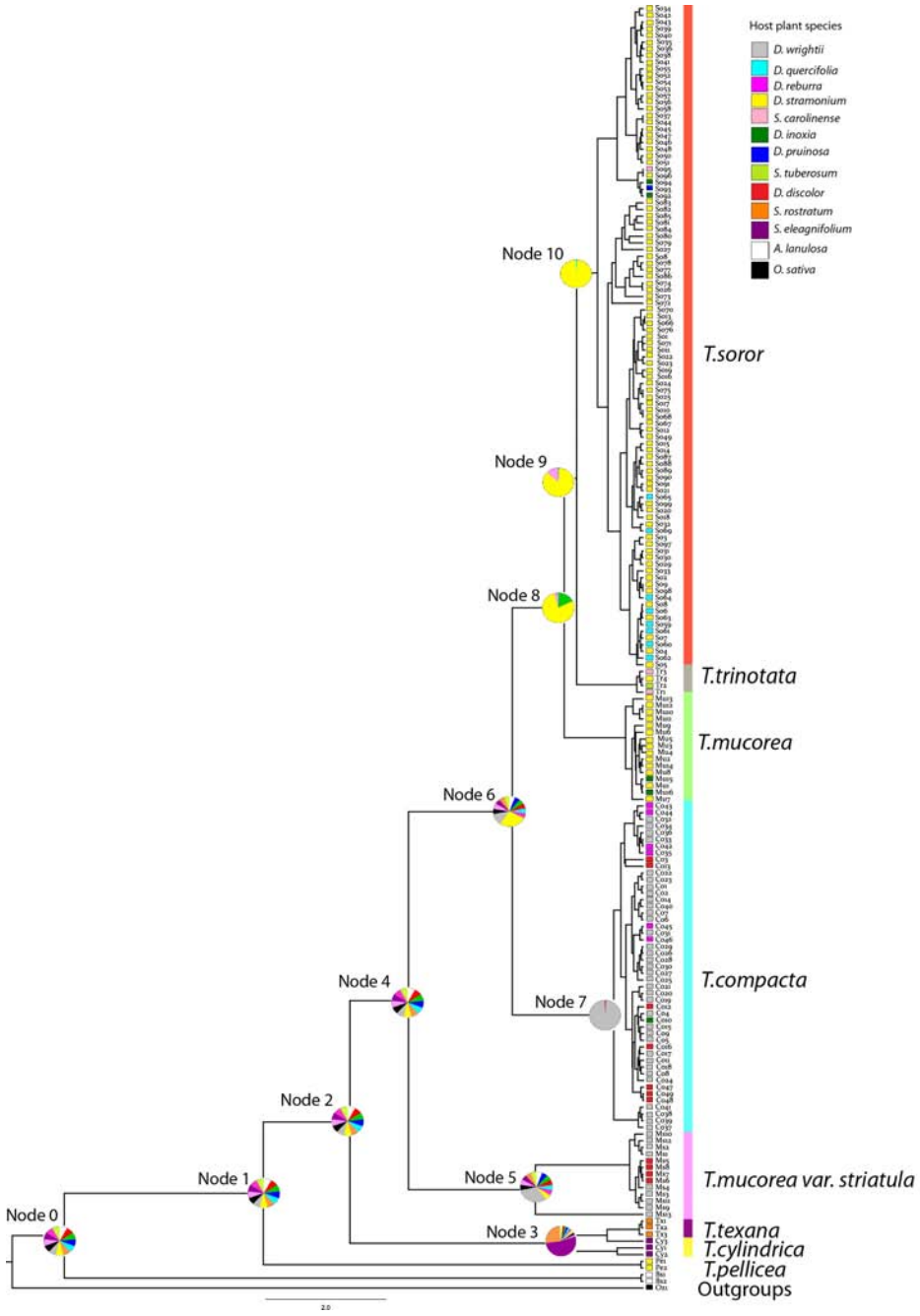


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Figure 8. Estimation of the ancestral host plant used to oviposition by *Trichobaris* species. The estimation was carried out in each node of *Trichobaris* haplotype tree using maximum likelihood, the pie charts illustrate the relative likelihoods of each of 13 possible host plants. The colored box at the end of each terminal are the plants where the haplotype was collected, each *Trichobaris* species is indicated by lateral bars at right side.

### 4.1 *Trichobaris* species and Barber's key

The following characters in Barber's key (Barber, 1935) are critical to distinguish among *Trichobaris* species: female rostrum length, body shape and male genitalia. In Barber' key female rostrum allows to distinguish *T. soror, T. pueblana* and *T. major* from all others *Trichobaris* species. In our study, in fact, we found that male and female rostrum shape is an important character that has phylogenetic signal in *Trichobaris* species (Supplementary Material Fig. S1), whereas body shape is highly convergent probably because among weevils, body shape in weevils can be influenced by different environmental conditions (Lemic, et al. 2015). The rostrum has been identified has a key innovation for the evolution of weevils (Oberprieler *et al.* 2007) also at a lower taxonomic level, in this genus, it continues been and important trait for the evolution of the group (Figure 4).

We found evidence that the species *T. soror, T. major, T. trinotata, T. compacta, T. texana and T. cylindrica* are distinct in form each other (Fig. 3). In others species, such as *T. insolita* and *T. championi* the clustering of individuals had no statistical significance, although this might be a consequence of small sample size (Supplementary Material, Table S1).

We found that *T. soror* and *T. major* are morphologically distinguishable (Fig. 3) but they do not show differences in COI haplotypes (Fig. 3). *Trichobaris mucorea* and *T. mucorea* var. *striatula* show close morphological clustering (Fig. 3), but they are genetically very distant, in fact, *T. mucorea* is closely to *T. soror* whereas *T. mucorea* var. *striatula* is closer to *T. compacta* (Fig. 3 - 8).

Species of *Trichobaris* missing in the phylogeny were *T. pueblana, T. championi, T. bridwelly* and *T. insolita.* Even though we sampled several populations in the state of Puebla and in the reported host plant *(D. stramonium),* we did not find *T. pueblana*. *Trichobaris championi* is a crop pest of tomatillo (*Physalis ixocarpa*) in Mexico (Calyecac-Cortero et. al 2004). Nevertheless, the only collected weevils on wild *Physalis* species was *T. mucorea,* which has also been reported on *Nicotiana attenuata* stems (Diezel, et al. 2011). *Trichobaris bridwelly* was reported by Barber as the single weevil species that feed into *D. stramonium* fruits in the USA (Barber, 1935); however, the *Trichobaris* species collected by us on *D. stramonium* in the USA was *T. soror* (see details below in text). Finally, *T. insolita* has only been reported for a single locality in Florida (USA), on a *Physalis sp*. patch (Barber, 1935). We were not able to corroborate this finding.

### 4.3 Phylogeographic patterns and host plant associations

Although our sampling of *Trichobaris* species did not cover all the geographic reported for USA's midwest (*T. texana, T. bridwelly* and *T. trinotata;* Barber 1935; Cuda and Burke, 1984), important findings about these species are reported in terms of their host plant. For example, whereas *T. bridwelly* was reported as the only weevil associated to *D. stramonium* in the USA (Barber, 1935), we found that the insects collected on *D. stramonium* in the USA (locality 1VA, Fig.1 and Table 1); So93, So94, So95 and So96) vary only in 2 or 3 mutational steps on the *T. soror's* haplotype network (Fig. S9; Supplementary Material). Thus, there is a possibility that *T. soror* is also distributed in western USA. The reported potato pest, *T. trinotata,* is associated to *S. tuberosum* and to *S. carolinense* (Cuda and Burke, 1986; Wise, 2007), and our study supports these observations (Fig. S10; Supplementary Material).

Host plant designed on haplotype network and ancestral host estimation on the phylogeny showed several interesting aspects (Fig. 8). First, the use of *Solanum* plants by Trichobaris weevils could be older than the occupation of *Datura,* except for *S. carolinense* and *S. tuberosum* whose estimated ancestral host is *D. stramonium*. Second, the occupation of different species of *Datura* by *T. compacta* may be more recent than the interaction *with D. wrightii*. Almost all beetles collected in *D. stramonium* were *T. soror*, which also colonize *D. quercifolia. T. mucorea* is associated with *D. stramonium,* and *T. mucorea* var. *striatula* is associated with *D. wrightii* and with a clade within *D. discolor*. Inconclusive cases, possibly due to sample size are *T. trinotata, T. texana, T. cylindrica* and *T. pellicea*. This means that historical associations and recent colonizations are both important in explaining *Trichobaris* relationship with its host plants.

A possible directionality on the evolution of resource use in weevils has been proposed (Malvardi et al., 2002). For *Trichobaris* species, we found that the use of stems and fruits is bidirectional. In the case of *T. trinotata*, we found that this species develops in the stems of both *S. carolinense* and *S. tuberosum*. It is likely that their ancestor developed in *Datura* fruits (Fig. 8).

It has been suggested that host shifts of closely related insect species are more strongly correlated with host plant defensive secondary chemistry than plant with plant phylogeny (Futuyma & Agrawal, 2009: Becerra, 2015). In the case of *T. soror* it is known that there is spatial variation in the levels of infestation of *D. stramonium* (Borbolla, 2015), likely associated to the alkaloid

production, namely atropine and scopolamine (Miranda *et al.*, 2016). Futures studies are needed to investigate the role of secondary compound chemistry in the association of *Trichobaris* with its plant hosts.

## 5 Conclusions

Phylogenetic relationships and geometric morphology of *Tichobaris* species show that *T. soror* and *T. major* are different in morphological grouping but they are the same species and that among *T. mucorea* and *T. mucorea var. striatula* they are close in morphological grouping but they are different species. Haplotype phylogeny suggests that the species could have emerged recently through coevolution and colonization to their host plants. Our results indicate that both processes were important in the evolution of species *Trichobaris* in relationship with its plant host.


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**Compliance with ethical standards**

**Conflict of interest**. Authors do not have any financial relationship with the organization that funded the research. The authors declarate they have no conflict of interest.

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**Supplementary material. Research article 3**

Figure S1. Discriminant analysis by species pair.

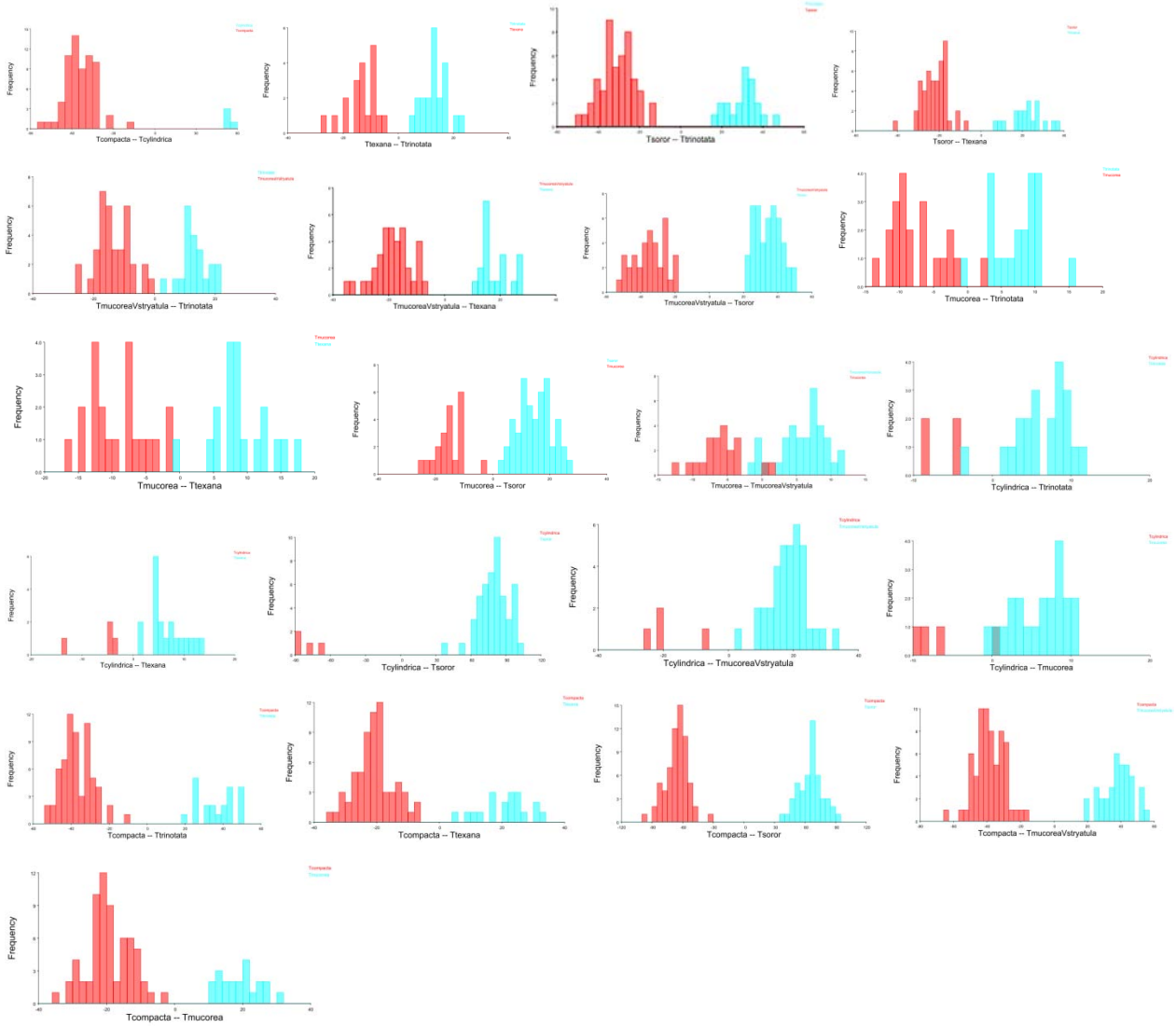
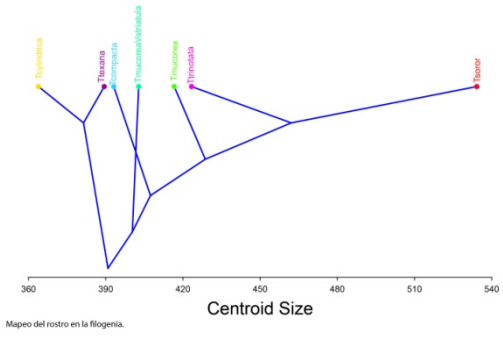
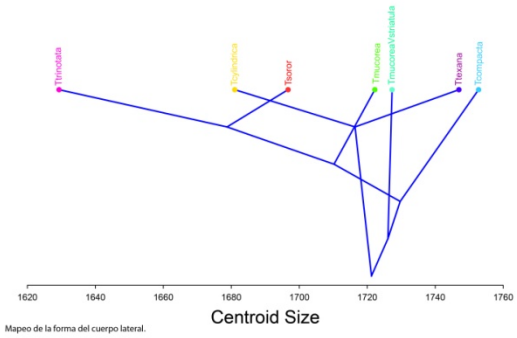


Table S1 Statistical significance of discriminant analysis.

| | | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstry | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tsoror | | | | | | | | | | |
| Proclusters distance | | | | | | | | | | | |
| Mahalanobis distace | | | | | | | | | | | |
| T2 p(parametrica) | | | | | | | | | | | |
| Proclusters distance (permutation Test) | | | | | | | | | | | |
| T2 (permutation Test) | | | | | | | | | | | |
| | Tmajor | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.0973 | | | | | | | | | |
| Mahalanobis distace | | 4.6259 | | | | | | | | | |
| T2 p(parametrica) | | 339.9722 p( 0.9996) | | | | | | | | | |
| Proclusters distance (permutation Test) | | <.0001 | | | | | | | | | |
| T2 (permutation Test) | | <.0001 | | | | | | | | | |
| | Ttrinotata | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.1453 | 0.1728 | | | | | | | | |
| Mahalanobis distace | | 7.7600 | 6.4953 | | | | | | | | |
| T2 p(parametrica) | | 874.0463 p( 0.9710) | 472.5151 p( 0.9457) | | | | | | | | |
| Proclusters distance (permutation Test) | | <.0001 | <.0001 | | | | | | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | | | | | | | | |
| | Tcompacta | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.0965 | 0.1419 | 0.1118 | | | | | | | |
| Mahalanobis distace | | 11.3534 | 8.6307 | 8.6172 | | | | | | | |
| T2 p(parametrica) | | 3493.0470 p(0.9321) | 1300.1753p( 0.9807) | 1174.1328 p( 0.9824) | | | | | | | |
| Proclusters distance (permutation Test) | | <.0001 | <.0001 | <.0001 | | | | | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | <.0001 | | | | | | | |
| | Tmucorea | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.0975 | 0.1374 | 0.0806 | 0.0447 | | | | | | |
| Mahalanobis distace | | 5.4887 | 5.9096 | 3.8451 | 6.1361 | | | | | | |
| T2 p(parametrica) | | 437.2619 p( 0.9976) | 391.1399 p( 0.9648) | 155.2381 p( 0.9974) | 595.3353 p(0.9990) | | | | | | |
| Proclusters distance (permutation Test) | | <.0001 | <.0001 | 0.0030 | 0.1250 | | | | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | <.0001 | <.0001 | | | | | | |
| | TmucoreaVstryatula | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.0885 | 0.1360 | 0.1055 | 0.0455 | 0.0380 | | | | | |
| Mahalanobis distace | | 7.9485 | 7.6938 | 4.9226 | 8.6139 | 3.2868 | | | | | |
| T2 p(parametrica) | | 1224.8739 p(0.9713) | 822.5013 p( 0.9397) | 310.9771 p( 0.9952) | 1615.5649, 0.9799 | 138.6366 p(0.9999) | | | | | |
| Proclusters distance (permutation Test) | | <.0001 | <.0001 | <.0001 | 0.0260 | 0.5480 | | | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | <.0001 | <.0001 | 0.0120 | | | | | |
| | Ttexana | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.0941 | 0.1400 | 0.1405 | 0.0622 | 0.0797 | 0.0586 | | | | |
| Mahalanobis distace | | 6.5127 | 6.1384 | 5.1615 | 6.2862 | 4.2670 | 5.8767 | | | | |
| T2 p(parametrica) | | 573.8917 p( 0.9904) | 399.5820 p(0.9533) | 265.7452 p(0.9748) | 578.9467 p( 0.9988) | 181.6148 p( 0.9924) | 416.4208 p(0.9822) | | | | |
| Proclusters distance (permutation Test) | | <.0001 | <.0001 | <.0001 | 0.0020 | 0.0020 | 0.0500 | | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | <.0001 | <.0001 | 0.0010 | <.0001 | | | | |
| | Tcylindrica | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.1426 | 0.1911 | 0.0704 | 0.1074 | 0.0829 | 0.0997 | 0.1288 | | | |
| Mahalanobis distace | | 12.6186 | 7.7918 | 3.5491 | 10.5492 | 3.4788 | 5.7858 | 3.6494 | | | |
| T2 p(parametrica) | | 586.9634 p (0.9514) | 208.1580 p( 0.9169) | 42.3230 p( 0.9982) | 418.9605 p(0.9980) | 40.6640 p(0.9985) | 119.4252p( 0.9971) | 44.0072 p( 0.9953) | | | |
| Proclusters distance (permutation Test) | | <.0001 | 0.0010 | 0.6640 | 0.0370 | 0.3090 | 0.1180 | 0.0100 | | | |
| T2 (permutation Test) | | <.0001 | <.0001 | 0.1610 | <.0001 | 0.2720 | <.0001 | 0.1690 | | | |
| | Tchampioni | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.1185 | 0.1807 | 0.1739 | 0.1044 | 0.1243 | 0.1121 | 0.1099 | 0.1653 | | |
| Mahalanobis distace | | 8.2871 | 6.7867 | 5.1899 | 8.8388 | 5.1873 | 9.8317 | 5.7120 | 2.2679 | | |
| T2 p(parametrica) | | 193.6656 p(0.9988) | 122.8255 p (0.9669) | 70.7049 p 0.9842 | 223.88 p(0.999) | 70.6329 p(0.9843) | 265.8227 p(0.9554) | 84.5336 p (0.9582) | 8.8175 p (0.8470) | | |
| Proclusters distance (permutation Test) | | 0.0210 | 0.0040 | 0.0010 | 0.1110 | 0.0430 | 0.1040 | 0.1230 | 0.0650 | | |
| T2 (permutation Test) | | 0.0010 | <.0001 | 0.0160 | 0.0130 | 0.0200 | <.0001 | 0.0040 | 0.1350 | | |
| | Tinsolita | Tsoror | Tmajor | Ttrinotata | Tcompacta | Tmucorea | TmucoreaVstryatula | Ttexana | Tcylindrica | Tchampioni | Tinsolita |
| Proclusters distance | | 0.1932 | 0.2046 | 0.1499 | 0.1781 | 0.1563 | 0.1803 | 0.2009 | 0.1442 | 0.2558 | |
| Mahalanobis distace | | 10.7831 | 6.8155 | 6.1204 | 11.5087 | 6.2601 | 9.9831 | 5.1299 | 1.2309 | 2.2267 | |
| T2 p(parametrica) | | 113.8529p( 0.9999) | 44.5931 p( 0.9978) | 35.7563 p(0.9968) | 130.4122 p( 1.0000) | 37.4079 p(0.9962) | 96.7311 p( 0.9973) | 24.9997p(0.9980) | 1.2121 p( 0.9277) | 3.7186 p( 0.5914) | |
| Proclusters distance (permutation Test) | | 0.0160 | 0.1010 | 0.5570 | 0.1430 | 0.2820 | 0.1640 | 0.0020 | 0.3970 | 0.2470 | |
| T2 (permutation Test) | | 0.0890 | 0.1350 | 0.2260 | 0.2080 | 0.3920 | 0.0230 | 0.4200 | 0.4130 | 0.2470 | |

642 Figure S3. Phylogenetic signal at morphogeometric analysis.

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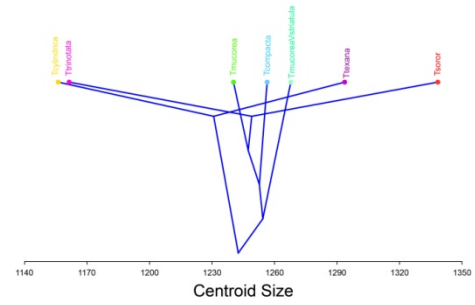


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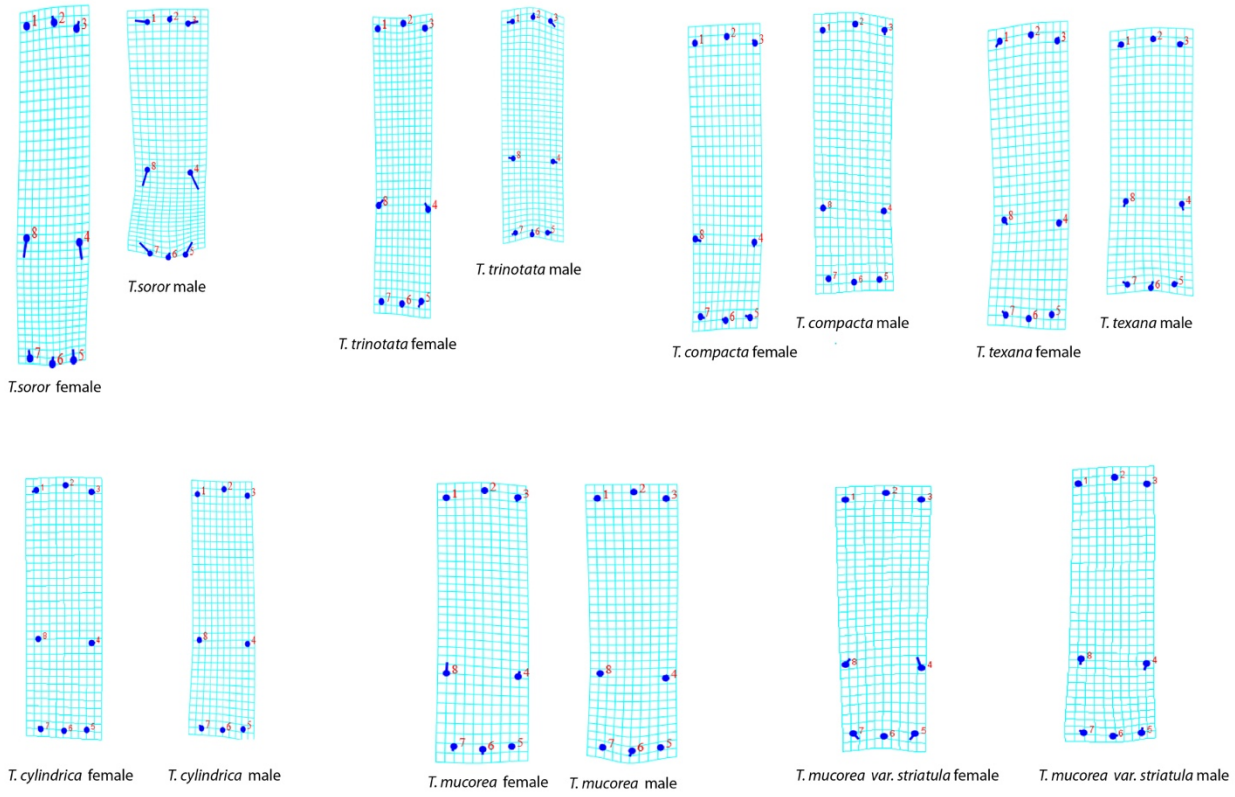
647

648 Table 2S. Phylogenetic signal

| **Permutation test again the null hypothesis of no phylogenetic signal for centroid size** | |
|---|---|
| **Body shape** | p: 0.9902 |
| **Lateral view** | p: 0.3758 |
| ***Pronotum*** | p: 0.9843 |
| ***Rostrum*** | p: 0.0243* |

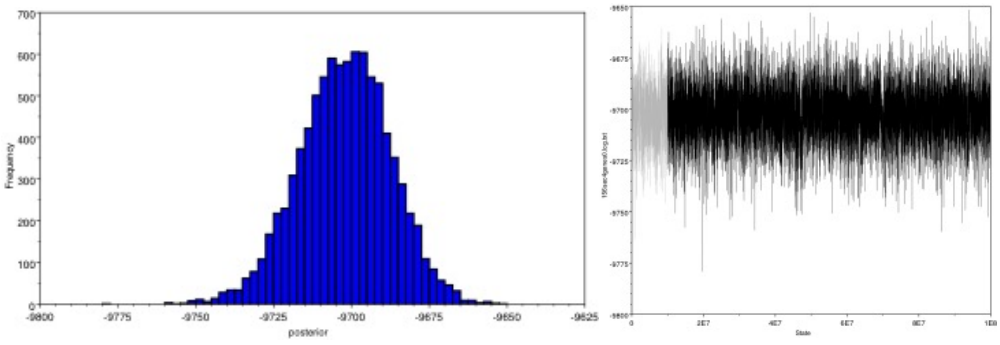
649 (*) statistically significant




650

651 Figure S3. Grill morphometric geometric analysis of changes in rostrum shape of each species of
652 *Trichobaris*.

653




654

655 Figure S4. Posterior probability of parameters and tracer for the calculation of *Trichobaris*
656 phylogeny using four genetic markers, and 156 insects.

657

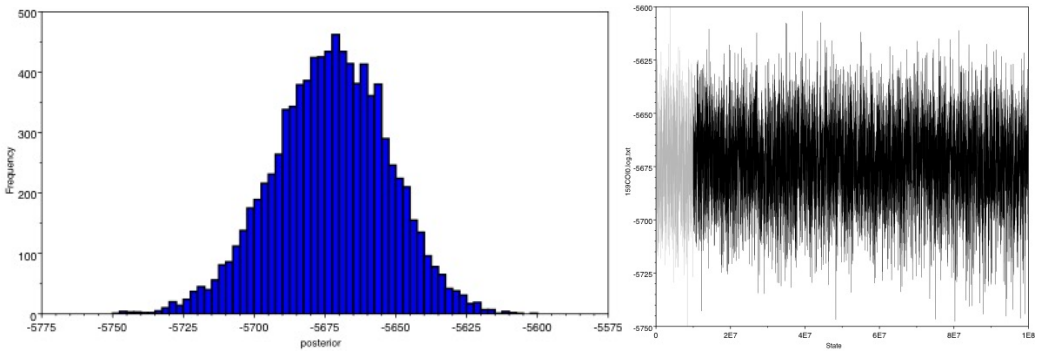



Figure S5 Posteriors probability of parameters and tracer for the calculation phylogeny using COI haplotypes for *Trichobaris* species.

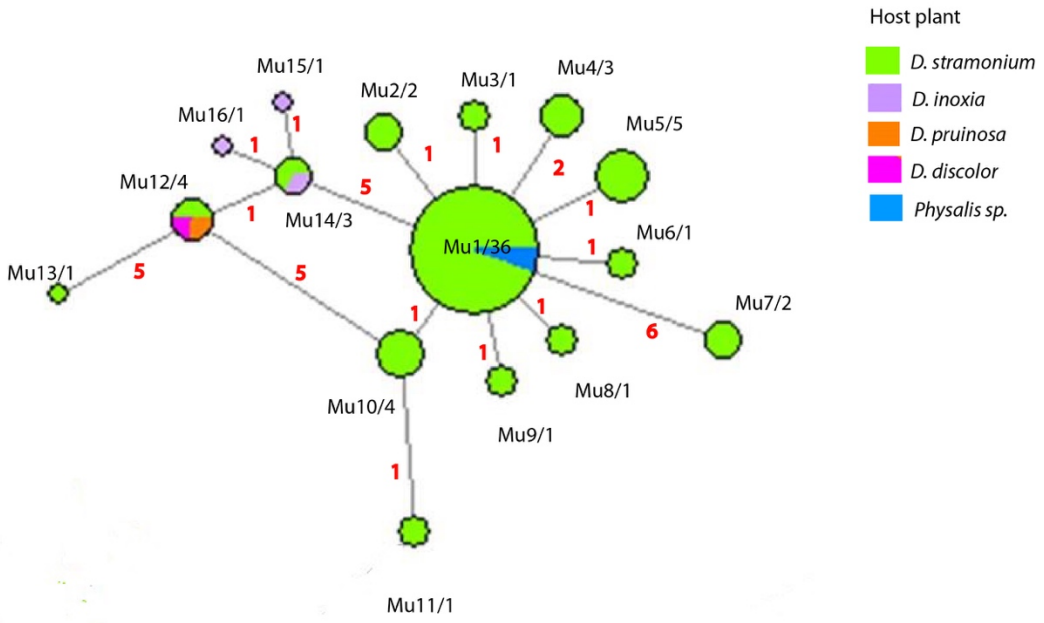



Figure S8. Haplotype network of *Trichobaris mucorea*. Obtained with the variation of the COI (663pb) gene by "Median joining" algorithm. Red numbers represent mutational changes.

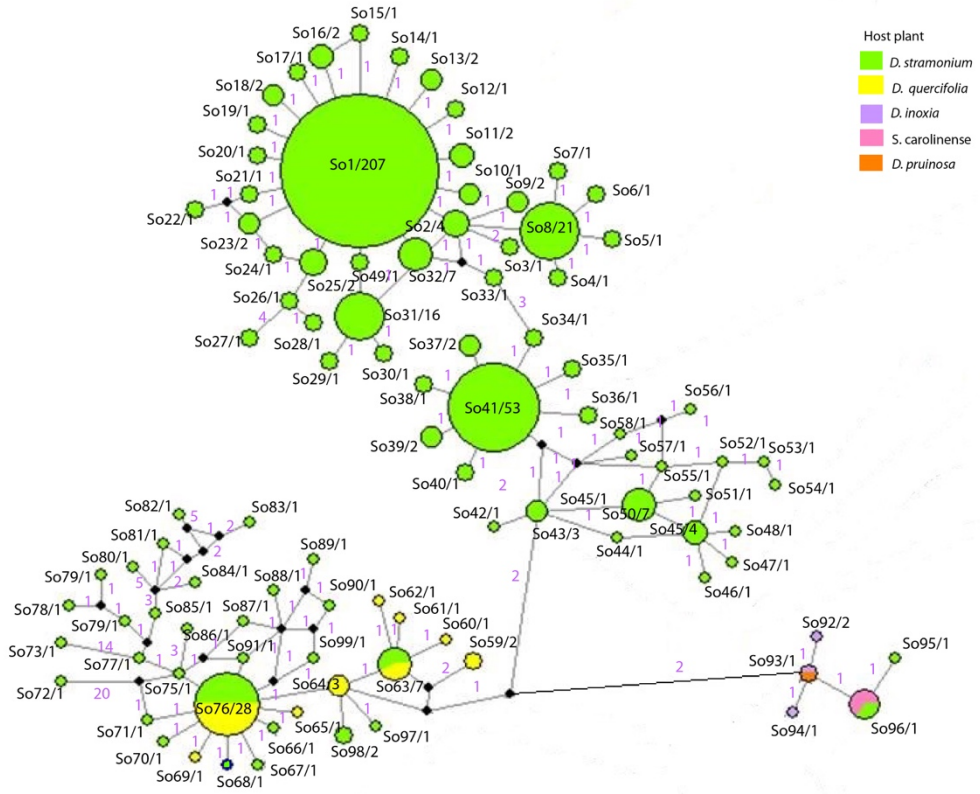
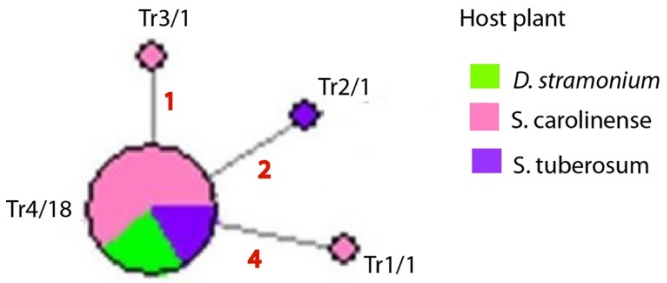
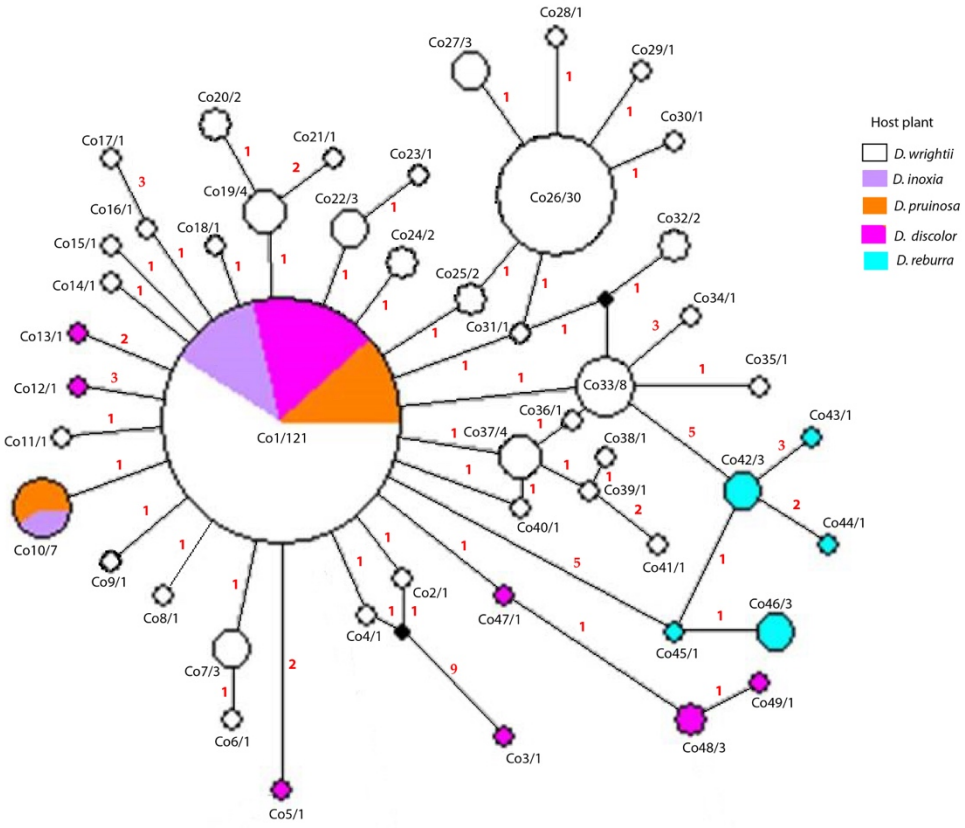



Figure S9. Haplotype network for *Trichobaris soror*. Obtained with the variation of the COI (663pb) gene by the algorithm "Median joining" the black dots represent vectors and purple numbers the number of mutational changes (haplotypes So1 to So42 previously reported on De-la-Mora, et. al. 2015).



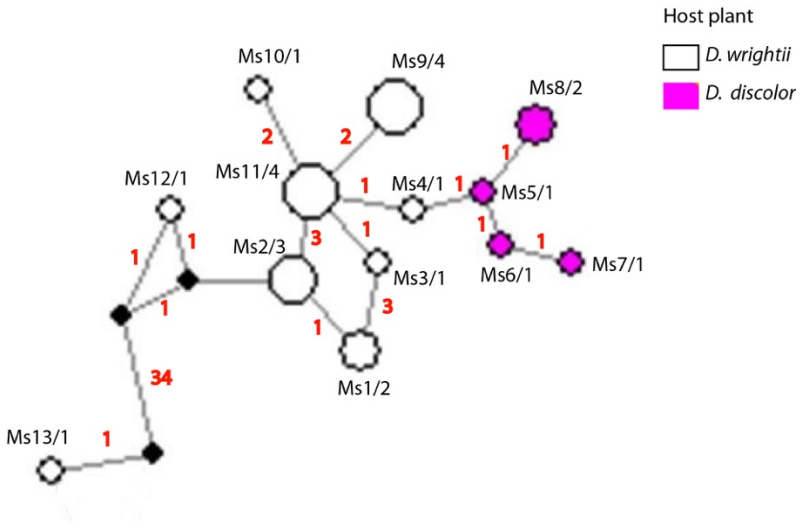

675

676 Figure S10. Haplotype network of *Trichobaris trinotata*. Obtained with the variation of the COI
677 (663pb) gene by the "Median joining" algorithm. Black dots represent vectors and numbers on
678 branches represent mutational changes.




679

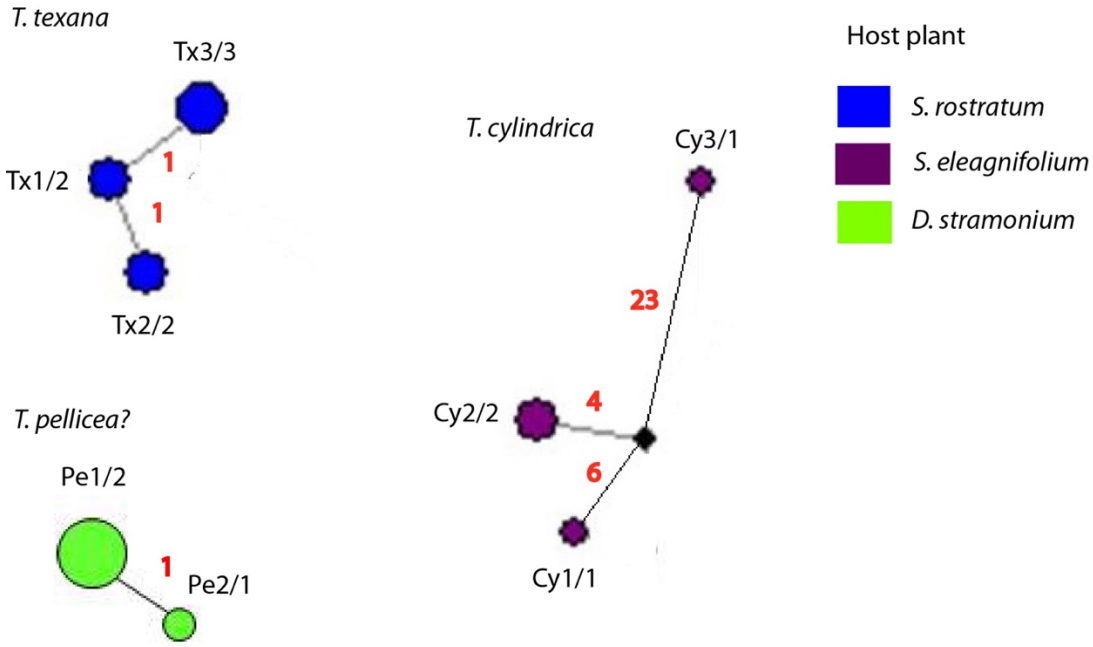
680 Figure S11. Haplotype network of *Trichobaris compacta*. Obtained with the variation of the COI
681 (663pb) gene by the "Median joining" algorithm. Black dots represent vectors and red numbers
682 mutational changes (from De-la-Mora in press).




683

684

685 Figure S12. Haplotype network of *Trichobaris mucorea var. striatula*. Obtained with the variation
686 of the COI (663pb) gene by the "Median joining" algorithm. Black dots represent vectors and red
687 numbers the number of mutational changes.




688

689 Figure S13. Haplotypes networks: *Trichobaris texana* (purple), *Trichobaris cylindrica* (yellow), and
690 *Trichobaris pellicea* (*) (white). Build with the genetic variation on COI gene (663pb) using the
691 "Median joining" algorithm. Black dots represent vectors and red numbers mutational changes. *
692 Sampled in stage larva.

## DISCUSIÓN GENERAL

En este estudio se analiza la especiación en curculiónidos del género *Trichobaris* asociados a plantas del género *Datura*, integrando información filogeográfica, filogenética, morfológica y de mapeo de la planta huésped. Los tres capítulos de la introducción nos permiten situar en un contexto más amplio las preguntas en el estudio de la evolución de los curculiónidos que se contestan con los tres artículos de investigación.

Como se ha expuesto en el capítulo tres (*Speciation in weevils*), la amplia diversidad de curculiónidos se debe a las innovaciones evolutivas del grupo, así como a sus asociaciones con hongos, gimnospermas y angiospermas (Anderson, 1993; Oberprieler *et al.* 2007), siendo estas últimas de mayor influencia para la diversificación de los curculiónidos (Farrell, 1998; Hunt *et al.* 2007; McKenna *et al.*, 2009).

La evidencia sobre evolución de curculiónidos y sus plantas huésped se aborda en el capítulo dos (*Insect speciation and plant interaction*), donde se señala y ejemplifica que la naturaleza misma de la interacción debe ser considerada para el análisis e interpretación de los patrones macro- y micro-evolutivos en el estudio de la diversificación de los curculiónidos.

En el capítulo uno (*Phylogeographic and phylogenetic patterns of speciation*), se describen de manera general los modelos de especiación, para situar el estudio de la diversificación de los curculiónidos en relación a la planta huésped como el factor de selección divergente (Especiación Ecológica, Nosil, 2012), lo que implica que la especiación está asociada a cambios en la planta huésped. No obstante, no se descartan otras presiones selectivas, como apareamiento no aleatorio (Johnson, 1982), competencia con otros herbívoros o depredación (Bernays & Graham, 1988), debido a la complejidad de fenómenos asociados con la especiación (e.g. Supple *et al.* 2014).

Mi enfoque abordó la hipótesis de que la planta huésped influyó en la variación genética del género *Trichobaris* en dos niveles evolutivos: A nivel microevolutivo las especies especialistas tendrán menos variación genética y ésta se encontrará asociada a la planta huésped, contario a especies generalistas donde se espera mayor variación genética y no asosciación genética en las diferentes especies de plantas huésped. A nivel macroevolutivo, las especies de *Trichobaris* especialistas de huésped mostrarán clados asociados a plantas huésped recientes mientras que las generalistas

mostrarán paralelismos asociados a plantas huésped recientes. A continuación se discutirá cada uno con base en los resultados de este estudio.

**A nivel microevolutivo**

Los dos primeros artículos de investigación describen la variación genética de dos especies de *Trichobaris* ampliamente distribuidas, que varían en su grado de especificidad a la planta huésped. La primer hipótesis de esta tesis es que la variación genética de la especie especialista sería menor que en la generalista. En este estudio no encontramos evidencia para sostener esta hipótesis al comparar *T. soror* y *T. compacta,* ya que ambas presentaron variación genética similar (*h*= 0.709 y 0.663, respectivamente). Esto puede obedecer a tres razones, entre otras: a que el marcador utilizado para medir la variación genética no refleja los efectos de la especialización (Harrison, 1991), debido a que es neutral o cuasi-neutral, a que no existe dicha reducción de la variación genética debida a la especialización (i.e., plasticidad fenotípica; Nylin & Gotthard, 1998; Agrawal, 2001), o a que la presión selectiva que ejerce la planta sobre el curculiónido no es tan fuerte como para reducir la variación genética. Dado que el proceso de especiación es muy complejo y en él pueden estar involucradas varias presiones selectivas en distintos estados del desarrollo de los insectos, no se descarta una reducción de la variación genética en la especialización al agente selector de mayor intensidad.

En el caso de la estructuración geográfica entre especialistas y generalistas, las especies especialistas podrían estar reflejando patrones de variación genética moldeados por los mismos factores que también ocurrieron en sus plantas asociadas a lo largo de su distribución, como es el caso del *Curculio hilgendorfi,* y no así para las especies generalistas como *Curculio sikkimensis* (Aoki *et al.* 2011; Toju & Sota, 2006). En nuestro estudio, *T. soror* muestra dos grupos genéticos asociados a la Faja Volcanica Transmexican y a la Sierra Madre Sur, en concordancia con lo reportado en otros curculiónidos que se distribuyen en la misma región geográfica (Sánchez-Sánchez *et. al*. 2012; Anducho-Reyes *et al.* 2008), no obstante, es necesario describir la variación genética de *Datura stramonium* en esta área. El único estudio a la fecha (Andraca, 2009), no incluye poblaciones de la Sierra Madre Sur. En el caso de *T. compacta* no encontramos estructuración genética geográfica a lo largo de las poblaciones muestreadas; es decir, no se encontraron grupos genéticos. Tampoco se

encontró variación genética asociada a plantas huésped, porque aún cuando algunos haplotipos fueron muestreados sólo en una o dos especies exclusivamente, no presentaron una distancia genética mayor a un paso mutacional entre ellos en la red de haplotipos. También, el haplotipo más frecuente fue muestreado en cuatro hospederos distintos, lo que significa que la migración entre plantas huésped ha homogenizado la variación genética a lo largo de la distribución de *T. compacta,* o bien que la colonización a estas plantas huésped es muy reciente, por lo que no se encuentran distintos grupos genéticos, como se ha reportado para otros taxa que se distribuyen en la misma área (Devitt, 2006; Sullivan, *et al.* 1997).

**A nivel macroevolutivo**

En el tercer artículo de investigación se estimó la filogenia de *Trichobaris* y se abordaron las hipótesis macroevolutivas en la diversificación de curculiónidos (clados asociados o paralelismos entre las especies de *Trichobaris* y sus plantas huésped). La filogenia muestra que las relaciones evolutivas estimadas con los marcadores moleculares son muy cercanas a las propuestas de Barber (1935). Dos hallazgos de este estudio son: (1) que *T. soror* y *T. major,* a pesar de ser distintas morfológicamente (en tamaño, color y forma), presentan la misma secuencia haplotípica de COI, lo que sugiere que son la misma especie. Y (2) que *T. mucorea* y *T. mucorea* var. *striatula,* a pesar de ser similares morfológicamente, están distantes entre sí en la filogenia y la red haplotípica.

En curculiónidos muchas, si no es que la mayoría, de las angiospermas sirven como planta huésped para el desarrollo de la larva; los linajes de curculiónidos muestran un alto grado de asociación con un limitado rango de plantas huésped, haciendo que los eventos de coespeciación sean altamente probables (Anderson, 1993). La hipótesis propuesta es que las especies especialistas mostrarán clados asociados a plantas huésped, mientras que las generalistas mostrarán paralelismos asociados a plantas huésped, pues es más factible que reflejen colonizaciones. Un evento de colonización ocurre cuando la diversificación ocurre previa al establecimiento en una nueva área (en este caso planta huésped). Para lo cual es necesario tener la filogenia calibrada y el tiempo de formación del área (e.g., Percy *et al*., 2004). Nosotros pusimos a prueba esta hipótesis en las especies del género *Trichobaris,* calibrando la filogenia de haplotipos de COI para tener una medida de la divergencia entre las especies, y reconstruimos el estado ancestral de la planta huésped para evaluar la historicidad de la interacción. Nuestro primer resultado al calibrar la filogenia de haplotipos, es que el género podría haber surgido hace unos 6 (±1) m. a., lo cual concuerda con lo señalado por

Anderson y O'Brien (1996), quienes señalan que los curculiónidos, como *Trichobaris*, distribuidos principalmente en la región Neártica y con pocas especies endémicas en México, podrían ser de un origen muy reciente. El mapeo de la planta huésped en la red de haplotipos y la reconstrucción del estado ancestral en la filogenia arroja varios resultados. Primero, que el uso de las plantas *Solanum* podría ser más antigua que la ocupación de las plantas del género *Datura*, a excepción de *S. carolinense* y *S. tuberosum* cuya reconstrucción del estado ancestral sería *D. stramonium*. Segundo, que la ocupación de distintas especies de *Datura* por *T. compacta* puede ser más reciente que la interacción con *D. wrightii*. Finalmente, en relación a la hipótesis de clados asociados o paralelismos entre las especies de *Trichobaris* y sus plantas huésped, se puede inferir que ambos procesos fueron importantes en la evolución de las especies de *Trichobaris.* Casi todos los escarabajos colectados en *D. stramonium* son *T. soror*; aquí los paralelismos se presentan al colonizar *D. quercifolia*. Mientras que *T. compacta* muestra paralelismos en todas las especies de *Datura* donde fue colectada. *T. mucorea* es un clado asociado a *D. stramonium,* y *T. mucorea* var. *striatula* asociado a *D. writgthii,* con un clado en *D. discolor*. Los casos no concluyentes, posiblemente debido al tamaño de la muestra, son *T. trinotata, T. texana, T. cylindrica y T. pellicea.*

Como conclusión, de acuerdo a la hipótesis planteada de que las especies especialistas mostrarán clados asociados a plantas huésped, mientras que las generalistas mostrarán paralelismos asociados a plantas huésped no se sostiene en las especies de *Trichobaris*. Nosotros no encontramos un patrón de conservadurismo de la interacción entre clados asociados o paralelismos en especies con distinto grado de especificidad a sus plantas huésped; por lo que deducimos que ambos procesos fueron importantes en la evolución de las especies *Trichobaris.*

## CONCLUSIONES GENERALES

*Trichobaris soror* es una especie especialista en *Datura stramonium,* ampliamente distribuida en México y posiblemente en Estados Unidos. En México ha sufrido una expansión poblacional en su distribución sobre la Faja Volcánica Transmexicana (FVT); y se encuentra geográficamente estructurada en dos grupos, uno en la FVT y el otro en la Sierra Madre Sur.

*Trichobaris compacta* es una especie generalista que se desarrolla en *Datura wrightii, D. discolor, D. reburra, D. inoxia* y *D. pruinosa.* A lo largo de su distribución en México y Estados Unidos no

encontramos estructuración geográfica, pero sí una expansión poblacional. A excepción de *D. wrightii* sólo pocos haplotipos fueron exclusivos de una especie de planta huésped.

La filogenia género *Trichobaris* concuerda con las relaciones establecidas para las descripciones taxonómicas hechas por Barber (1935). La variación genética y el análisis de morfometría geométrica indica que *T. soror* y *T. major* podrían ser la misma especie, mientras que *T. mucorea* y *T. mucorea* var. *stratula* en realidad podrían tener caracteres morfológicos convergentes.

Las especies del género *Trichobaris* podrían ser muy recientes (tiempo de origen menor de 6 m.a.) y la por lo tanto la colonización a distintas plantas huésped también. La reconstrucción ancestral y el mapeo de la planta huésped nos señala las interacciones que podrían estar conservadas entre las especies de *Trichobaris* y las especies de *Datura*.

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