Bedhomme, S.; Lafforgue, G.; Elena Fito, SF. (2012). Multihost experimental evolution of a plant RNA virus reveals local adaptation and host specific mutations. Molecular Biology and Evolution. 29(5):1481-1492. doi:10.1093/molbev/msr314.

Title: Multihost experimental evolution of a plant RNA virus reveals local adaptation and host specific mutations.

Submission as a research article.

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Keywords: experimental evolution, local adaptation, *Tobaco etch virus*, parallel evolution, full genome sequence, virulence evolution.

Running head title: multihost viral experimental evolution.

Abstract:

For multihost pathogens, simultaneous adaptation to various hosts has important implications for both applied and basic research. At the applied level, it is one of the main factors determining the probability and the severity of emerging disease outbreaks. At the basic level, it is thought to be a key mechanism for the maintenance of genetic diversity both in host and pathogen species. Using *Tobacco etch potyvirus* (TEV) and four natural hosts, we have designed an evolution experiment whose strength and novelty are the use of complex multicellular host organism as hosts, and a high level of replication of the different evolutionary histories and lineages. A pattern of local adaptation, characterized by a higher infectivity and virulence on host(s) encountered during the experimental evolution was found. Local adaptation only had a cost in terms of performance on other hosts in some cases. We could not verify the existence of a cost for generalists, as expected to arise from antagonistic pleiotropy and other genetic mechanisms generating a fitness trade-off between hosts. This observation confirms that this classical theoretical prediction lacks empirical support. We discuss the reasons for this discrepancy between theory and experiment in the light of our results. The analysis of full genome consensus sequences of the evolved lineages established that all mutations shared between lineages were host-specific. A low degree of parallel evolution was observed, possibly reflecting the various adaptive pathways available for TEV in each host. Altogether, these results reveal a strong adaptive potential of TEV to new hosts without severe evolutionary constraints.

Introduction

The environment of a pathogen, and consequently the source of selection pressures is, for the majority of its life-cycle, its host. The particular host environment presents different forms of heterogeneity, even on short evolutionary time scales: from different cell types within a tissue, to different species (Thomas et al. 2002). Multihost pathogens, which encounter the latter, more extreme case of heterogeneity, should be able to cope with different biotic conditions but also, due to their parasitic reproduction strategy, exploit different cellular mechanisms and resources for their own growth and reproduction. The adaptation to various hosts is supposed to be constrained by the fact that mutations advantageous in one environment tend to be disadvantageous in another environment (Gandon 2004).

Simultaneous adaptation to various environments has important implications both for applied and basic research. In recent years, emerging diseases have represented an important threat for public health and agriculture (Anderson et al. 2004; Woolhouse, Haydon, Antia 2005; Parrish et al. 2008). In many instances, an emerging disease appears when a pathogen jumps to another host it was not infecting (or no longer infecting) and adapts (or re-adapts) to it. The increasing threat imposed by emerging and re-emerging diseases should prompt the improvement of predictive models and the design of preventive strategies to control emerging disease outbreaks. To do so, we still need to understand the mechanisms that make pathogen populations able to spillover from their reservoirs, successfully infect a new naïve host, adapt to it, and spread among the population of the new host.

In terms of basic research, simultaneous adaptation to various environments has been an important topic in evolutionary biology for many years. It is thought to be one powerful mechanism for the maintenance of genetic diversity (Kawecki 1994) and to play a crucial role in triggering the divergence of incipient species (Via 1990). Indeed, one way a pathogen can get around the evolutionary constraints imposed by the multiplicity of hosts is specialization

(or local adaptation) to one host species or to a small group of host species within the host range. Host specialization can be the first step towards the evolution of host races and further towards speciation (e.g. Filchak, Roethele, Feder 2000). Local adaptation to one host is predicted to have a cost, manifested through worse performance of the locally adapted pathogen on a non-native host (Kawecki, Ebert 2004). A recent literature survey established that, when looked for, a significant pattern of local adaptation is usually found while the cost of adaptation is, at best, small and in many cases non-significant (Hereford 2009).

Taking into account the potential for local adaptation, for any multihost pathogen specific lineages are likely to fall along a gradient, which in principle ranges from absolute specialists, those pathogens only able to infect and reproduce in a single host, to absolute generalists, able to infect and reproduce equally well within any species of the host range. The position of a particular pathogen lineage along this gradient depends on: i) environmental factors, ii) the genetic diversity within each lineage and iii) the global fitness of each evolutionary strategy. First, the environmental factors affecting the evolutionary fate of a multihost pathogen are the frequency and distribution of each species of the host range, which strongly influence the migration rate between host species (Woolhouse, Taylor, Haydon 2001; Ravigne, Dieckmann, Olivieri 2009). For example, for a plant pathogen a monoculture field is clearly a situation favouring specialist genotypes, while a diverse ecosystem is expected to favour generalist genotypes. An additional environmental factor for vector-transmitted pathogens is the availability and the specialization of the vectors. Indeed, vectors usually feed on hosts and themselves have a preference towards one or more hosts, thus strongly influencing the probability of transmitting the parasite from one species to another (Woolhouse, Taylor, Haydon 2001). Second, the genetic diversity of a pathogen population within one host species conditions the chances of appearance and selection of mutations that allow for a sufficient infectivity and reproduction rate to maintain the pathogen within another

host species. The genetic diversity itself depends on population genetic parameters such as the mutation rate, the reproduction rate, the intra-host effective population size, and population structure. Third, generalist evolutionary strategies have been predicted to be disadvantaged for three distinct reasons: (1) antagonistic pleiotropy, *i.e.* the fact that mutational effects are negatively correlated between hosts, is thought to play a preponderant role in many cases (Via 1990; Fry 1996; Gandon 2004). (2) Since generalists alternate between hosts, some pathogen genes can be expressed during infection in one host and not expressed in another host. The absence of expression part of the time implies the absence of selection acting on these loci and the potential for accumulation of mutations, which will be deleterious when expressed in the other host (Kawecki 1994). (3) The alternation between hosts by generalists implies that selection pressures specific to a particular host act only part of the time, slowing down the rate of allele fixation and elimination compared to that of a specialist (Whitlock 1996). Field studies have shown the disadvantage of generalists in a diversity of systems (e.g. Poulin 1998, fish parasites; Poulin, Mouillot 2004, helminth parasites of birds; Malpica et al. 2006, viruses and weeds; Straub, Ives, Gratton 2011, endoparasitoids and aphids). In other systems, however, the generalist strategy seems to be favoured (e.g. Krasnov et al. 2004, ectoparasitic fleas and small mammals; Hellgren, Pérez-Tris, Bensch 2009, *Plasmodium* sp. and birds). This discrepancy between theoretical predictions and some field data is likely to be due to the influence of other factors (environmental and genetic diversity) and to the lack of universality of the costs of adaptation (Hereford 2009).

An efficient way to investigate the factors and mechanisms of specialization and the implications for the evolution of specialist and generalist strategies is to use experimental evolution (Kawecki, Ebert 2004). It allows explicitly addressing hypotheses about the role of particular ecological and genetic factors that promote or hinder local adaptation, maintaining other factors fixed. A number of experimental evolution studies on specialization and

123 generalist/specialist strategies have already been published using a variety of experimental
124 systems (e.g. Turner, Elena 2000, *Vesicular stomatitis virus* (VSV)/hamster, human or canine
125 cell; Magalhaes et al. 2009, spider mites/host plants; Legros, Koella 2010,
126 microsporidia/mosquito). Almost all experimental evolution studies show a pattern of host
127 specialization (Novella et al. 1999; Turner, Elena 2000; Cooper, Scott 2001; Greene et al.
128 2005; Wallis et al. 2007; Agudelo-Romero, de la Iglesia, Elena 2008; Coffey et al. 2008;
129 Magalhaes et al. 2009; Vasilakis et al. 2009; Legros, Koella 2010; Coffey, Vignuzzi 2011;
130 Deardorff et al. 2011), but only in some cases is specialization accompanied by a cost
131 (Weaver et al. 1999; Turner, Elena 2000; Wallis et al. 2007; Agudelo-Romero, de la Iglesia,
132 Elena 2008; Vasilakis et al. 2009; Legros, Koella 2010; Deardorff et al. 2011). Finally,
133 evidence for the cost of being a generalist are even less frequent (Coffey et al. 2008; Legros,
134 Koella 2010).

135 Among the existing experimental evolution studies, those using RNA viruses are
136 overrepresented. There are various reasons for this overrepresentation. First, RNA viruses are
137 likely to evolve in a reasonable experimental time because they have large population sizes,
138 fast replication rates, and a high mutation rate due to the absence of proofreading activity of
139 the RNA polymerase (Elena, Sanjuan 2007). Second, the small genome size allows access the
140 full genome sequence of the evolved lineages and to reveal the genetic changes that underlie
141 phenotypic evolution. Third, RNA viruses present a large diversity of evolutionary strategies.
142 Examples in animals range from highly specialized viruses, such as the *Poliovirus*
143 *(Picornaviridae)* which only infects humans, to the *Influenza A virus (Orthomyxoviridae)*,
144 able to infect hosts from distant phylogenetic groups (Wolfe, Dunavan, Diamond 2007).
145 Diversity is also found in RNA plant viruses, with some viruses such as the *Tobacco etch*
146 *potyvirus* (TEV), which is a relative specialist compared to the *Cucumber mosaic*
147 *cucumovirus*, known to infect members of 16 different plant families. In this context, a group

of RNA viruses that has attracted a lot of attention is the arboviruses (arthropod-born viruses), whose infective cycle includes obligate alternation between vertebrate and insect hosts. The constraints imposed by the evolutionary trade-offs have been proposed as a likely cause of their relative genetic homogeneity and constancy over time (Woelk, Holmes 2002).

It is important to note that the majority of these experimental evolution studies use cell cultures as "hosts". Cell cultures are simple, homogeneous environments, with no tissue structure and no immune pressure, and probably represent conditions where selection pressures are constant and unidirectional. Actually, it has been shown that local adaptation obtained by evolution in mammalian cell cultures cannot be extrapolated to the corresponding organism (Ciota et al. 2007). Moreover, experimental evolution conducted *in vivo* and in the corresponding cell culture lead to very different results (Coffey et al. 2008). Additionally, all the studies cited above compare the outcome of one evolutionary history alternating between two hosts with the two corresponding single-host evolutionary histories. It is thus possible that the results in the alternate evolutionary history are not due to alternation of host environment, but are idiosyncratic to this specific treatment. In other words, when technically and experimentally possible, it is desirable to have, on top of the technical "within evolutionary history" replication, different generalist and specialist evolutionary histories. This extra level of replication allows for more robust conclusions on the existence of a specialist-generalist trade-off for a particular pathogen. Among the published evolution experiments with viruses, only four were *in vivo* experiments (Wallis et al. 2007; Agudelo-Romero, de la Iglesia, Elena 2008; Coffey et al. 2008; Deardorff et al. 2011). Three of these studies were designed to evaluate the evolutionary constraints imposed by vector transmission on adaptation (Wallis et al. 2007; Coffey et al. 2008; Deardorff et al. 2011). The other study used only two hosts and did not contain any evolutionary treatment with host alternation (Agudelo-Romero, de la Iglesia, Elena 2008).

For the present study, we designed an evolution experiment to study local adaptation of TEV to four hosts within its natural host range. The hosts were complete plants, with all the complexity this completeness implies for the dynamics of viral infection. Moreover, we put a special emphasis on the replication of specialist and generalist evolutionary histories: four "single host" and three "alternate host" evolutionary histories were experimentally derived with a high "within history" replication level. With this experimental set up, we aimed at collecting data in conditions that were "as natural as possible" and would allow for drawing robust and broad conclusions on generalist and specialist strategies, as well as on the conditions for local adaptation. The infectivity and virulence characteristics of the experimentally evolved lineages were analysed and their full genome consensus sequences obtained. It has to be noted here that testing for local adaptation and the cost of adaptation requires measuring fitness or variables tightly associated to it. Here, the ideal approach would have been to evaluate the viral population growth rate, but this approach was hardly experimentally tractable and we decided to use infectivity and virulence as fitness proxies instead. Infectivity is usually a good variable to evaluate local adaptation because it is a clear component of pathogen fitness and it is monotonically related to it (Kawecki, Ebert 2004). On the other hand, virulence is usually under stabilizing selection, with intermediate levels of virulence corresponding to the fitness optimum (Frank 1993; Jensen et al. 2006; Fraser et al. 2007; de Roode, Yates, Altizer 2008). The existence of this fitness optimum potentially implies a non-monotonic relationship between virulence and fitness, depending on the portion of the selection gradient where the measures are taken. Moreover, it is possible that the virus has a different virulence optimum in the four hosts used, making the relation between virulence and fitness even more complex. Virulence is thus less suitable for evaluating local adaptation, although it is often a useful parameter to understand the implications of the evolutionary and coevolutionary processes in host-pathogen systems.

Material and methods

Virus and plants

Our model system is *Tobacco etch potyvirus* (TEV). TEV is a member of the *Potyvirus* genus within the *Potyviridae* family and has a moderately wide host range (Shukla, Ward, Brunt 1994). It has a positive sense single-strand RNA genome of 9.5 kb that encodes a large polyprotein, which is auto-catalytically cleaved into ten multifunctional mature viral proteins (Riechmann, Lain, García 1992). Recently, an overlapping ORF coding a small additional protein after frameshifting has been discovered (Chung et al. 2008). The genome replication in *Potyviridae* is performed by a virus-encoded RNA-dependent RNA-polymerase that lacks proofreading activity. TEV mutation rate is thus high, estimated to be around 10^{-5} to 10^{-6} mutations per site and per generation (Tromas, Elena 2010).

Plasmid pMTEV contains the TEV genome (Bedoya, Daròs 2010), and was a generous gift by Dr. J.A. Daròs. The TEV genome used to generate this clone has been isolated from *Nicotiana tabacum* (Carrington et al. 1993) and its sequence is published elsewhere (Carrasco et al. 2007). A stock of infected tissue was generated before starting the evolution experiment (see Supplemental material for details).

Four host species were used: *N. tabacum*, *Nicotiana benthamiana*, *Datura stramonium* and *Capsicum annuum*. They all belong to the *Solanacea* family and TEV produces systemic symptoms in all of them. For all the experimental steps, plants were maintained in a greenhouse at 25 °C and a 16 h photoperiod.

Viral accumulation in each host

Before starting the experimental evolution, viral accumulation in each of the four hosts was measured by RT-qPCR (see Supplemental material for details) to ensure that transmission took place when the viral load had reached a plateau and that the quantity of

225 virus used for transmission was equivalent for the four host species. This was done to equalize
226 the size of the transmission bottleneck in every experimental evolution lineage, and thus
227 differences between lineages could not be attributed to differential influence of genetic drift.
228 The obtained accumulation curve indicated that at 7 dpi, viral accumulation had
229 reached a plateau and that viral genomes accumulated at the same level in *N. tabacum*, *N.*
230 *benthamiana* and *D. stramonium* (around 2.2×10^7 viral RNA molecules per 100 ng of total
231 RNA) and at a lower level in *C. annuum* (3.2×10^6 viral RNA molecules per 100 ng of total
232 RNA).
233
234 *Experimental evolution*
235 The experimental evolution design contained seven evolutionary histories (figure 1).
236 In four of them, the viruses were serially passaged in the same host, hereafter denominated as
237 lineages Nb (*N. benthamiana*), Ds (*D. stramonium*), Ca (*C. annuum*) and Nt (*N. tabacum*). In
238 the three other lineages, the viruses were serially passaged on alternate hosts using the
239 following pairs: (*N. benthamiana*, *N. tabacum*– hereafter NbNt), (*N. tabacum*, *C. annuum* –
240 NtCa) and (*D. stramonium*, *C. annuum* – DsCa). The first type of evolutionary history
241 represents the conditions for the selection of a specialist strategy, whereas the second type is
242 an experimental approximation of conditions selecting for a more generalist strategy. Each
243 evolutionary history was replicated ten times.
244 To initiate the experimental evolution, a sap was prepared with 300 mg of infected
245 tissue (from the previously described stock) and 450 µL of inoculation buffer. For each
246 replicate, two plants were mechanically inoculated with 5 µL of this sap on one leaf. All
247 lineages were thus started with a genetically homogeneous viral population and *de novo*
248 mutation was the only source of raw material on which selection and genetic drift could act
249 (Elena, Lenski 2003). For the subsequent passages, at 7 dpi, the aerial part of one of the two

plants in each lineage was collected. If the two plants presented symptoms, the plant to collect was chosen randomly. If only one presented symptoms, this one was collected. In both cases, the inoculated leaf was removed and a sap was prepared with 300 mg of symptomatic leaf tissue in 400 µL of inoculation buffer if the infected tissue was from *C. annuum,* and 150 mg of infected tissue in 1 mL of inoculation buffer for the other host species, so that each infection was started with similar amounts of viral RNA. For each lineage, two plants were then inoculated on one leaf with 5 µL of sap. Fifteen serial passages were performed. One of the Ca lineages was lost during experimental evolution.

The experimental procedure removes the natural vector of TEV (aphids) and thus represents a simplified version of the virus life-cycle. This choice was made because using aphid transmission would have greatly reduced the number of plants we could have infected and consequently the replication level of and within evolutionary history. The evolutionary implications of this choice are discussed below.

Infectivity and virulence measurement

After the 15th passage, infected tissue was collected from each lineage and the viral RNA content measured by RT-qPCR (see Materials and Methods). The obtained quantification was used to prepare saps of equal viral RNA concentration. Each of these saps was mechanically inoculated (5 µL of sap on one leaf) on three plants of each of the four host species. This way, we had a complete crossed design, fully replicated three times. For practical reasons, the inoculation was spread over four days, with the replicate lineages within an evolutionary history split between the days. Additionally, each day three plants of each species were inoculated with inoculation buffer as non-virus controls, and another three plants were inoculated with a sap (at the same concentration of viral RNA as the evolved lineages) made with the TEV stock used to start the experiment, and representing the ancestor for all

the evolved lineages. Before inoculation, the aerial part of each plant was measured (from the basis of the stem to the apex) with a precision of 0.5 cm. At 21 dpi, each plant was checked individually and the presence of symptoms was noted, to then calculate the infectivity. The aerial part was measured with a precision of 0.5 cm and weighted (with a Kern 440-35N balance, Kern and Sohn Gmbh) with a precision of 10 mg. We define virulence as the degree of damage caused to a plant by viral infection, and it is negatively correlated with host fitness (Shaner et al. 1992; Sacristán, García-Arenal 2008). We calculated the virulence expressed in size as:

$$Vir_{size}(E_iH_j) = 1 - \Delta size(E_iH_j)/\Delta size(control)$$

where $Vir_{size}(E_iH_j)$ is the virulence expressed on size of the i^{th} replicate of evolutionary history E when inoculated on the j^{th} replicate of host H and $\Delta size$ is the difference in size between the day of infection and 21 dpi. A similar virulence index was obtained from the weight, $Vir_{weight}(E_iH_j)$. However, $\Delta weight$ cannot be calculated directly because it is impossible to weigh the plant before inoculation. We thus established the correlation between weight and size for each species for plants of the same age as the ones we inoculated using an independent cohort of healthy plants reared in the same conditions as the one used for infectivity and virulence measurements. Using the correlation for each species and the size at inoculation, we could estimate the weight at inoculation for each plant and thus estimate $\Delta weight$.

Genomic consensus sequence determination in the evolved lineages

Total RNA was extracted from infected tissue of the 69 experimentally evolved lineages. The TEV genome was amplified in three overlapping fragments and the sequencing of the amplification products was outsourced (see supplemental material for details). We obtained the consensus sequence from nucleotide 48 to nucleotide 9492, *i.e.* 99% of the full

genome and 100% of the coding sequence. The average coverage was 2.41. The genomes were assembled and the mutations were identified using the Staden 2.0.0b7 package.

Our estimates of virulence and infectivity were done at the population level and we did not explore the variability of these variables within each replicate lineage. For this reason, we also directly sequenced PCR amplified virus population cDNAs rather than sequencing multiple clones isolated from the population. This consensus sequencing approach allows for detection of the dominant nucleotide at each base position. When multiple clean sequencing reads showed clearly the presence of two peaks at one position, the lineage was considered to be polymorphic at that position. However, it is impossible to measure the frequency of each allele with this method and mutations could be present in the virus population at frequencies lower than the detection threshold for a chromatogram. The real within-population diversity is thus higher than the one reported here.

Results

Infectivity and virulence

The analysis of infectivity and virulence data was performed on a data set containing only the data from lineages that had at least one mutation compared to the initial sequence of the ancestral TEV infectious clone. This reduced the data set from 69 to 53 independent lineages (6 to 9 independent lineages per evolutionary history).

A nominal logistic model with evolutionary history, host plant and their interaction was built to analyse their effect on infectivity. The “evolutionary history replication” was nested within the “evolutionary history” factor. This analysis revealed that all factors had a significant effect on infectivity (table 1, figure 2). Figure 2 also shows the behaviour of the ancestral virus, not included in the previously described nominal logistic model. The infectivity of the ancestral virus is in the mid-low range of infectivities of the derived

lineages, except on *N. tabacum* where it has an infectivity of one. An ANOVA with the same factors as above was performed with the virulence expressed on size and on weight as variables. In this case, the replication within evolutionary history was taken as a random factor. The method used was restricted maximum likelihood. For the two indices of virulence, evolutionary history did not have a significant effect whereas host and the interaction between host and evolutionary history had a significant effect (table 2, figure S1). The significant interaction for the three considered variables indicates that the different evolutionary histories produced distinct infection characteristic in the four hosts. Figure S1 also shows the virulence pattern of the ancestral virus across the four hosts (data not included in the previously described ANOVAs): its virulence is in the mid-high range of those of the derived lineages.

Using the full data set of the derived lineages, we could also ask more specific questions: do we have a signature of host specialization? Does this depend on the specialist/generalist characteristic of the evolutionary history? To answer these questions, each evolutionary history was classified as "specialist" (one host) or as "generalist" (two hosts in alternation). Additionally, each inoculation was classified as "local" or "foreign": a local inoculation is an inoculation on a host present during the experimental evolution for this precise lineage (for example, NbNt is local on *N. benthamiana* and *N. tabacum*; and Nb is local on *N. benthamiana*), whereas a foreign inoculation is an inoculation on a host which was not present during the experimental evolution (for example, NbNt is foreign on *D. stramonium* and *C. annuum*; and Nb is foreign on *N. tabacum*, *D. stramonium* and *C. annuum*). A nominal logistic model was constructed with "host", "specialist/generalist" and "local/foreign" and their double and triple interactions as factors to determine the effect on infectivity. The "specialist/generalist" and all interactions containing it did not have any significant effect. "Host", "local/foreign" and their interaction had a significant effect (table 3) and the interaction was due to the fact that on *D. stramonium*, the local lineages had an

350 infectivity that was two times larger than the foreign ones (figure 3a). This effect was present,
351 to a much lower extent, on *N. tabacum* while on the two other hosts local and foreign lineages
352 had identical infectivity. Therefore, this result indicates a strong local adaptation of lineages
353 that have evolved all or part of the time on *D. stramonium*. An ANOVA with the same factors
354 was then performed to analyse the influence on the two virulence indices. For virulence
355 expressed on size, the statistical results were very similar to those for infectivity (table 4), but
356 the origin of the significant interaction was different (figure 3b): virulence expressed on size
357 was identical for local and foreign lineages on all hosts except on *C. annuum*, where the local
358 lineages had a higher virulence than the foreign ones. For virulence expressed on weight, the
359 factors "host" and "local/foreign" had a significant effect and no other effect was significant.
360 The "local/foreign" effect was due to a higher virulence of local lineages, particularly on *D.*
361 *stramonium* and *C. annuum* (figure 3c).
362 Another way of looking at local adaptation, which also allows determining whether a
363 cost of adaptation exists, is to perform analyses by pairs of single host evolutionary histories.
364 Indeed, with the complete factorial design used, the three variables (infectivity, virulence
365 expressed on size, and virulence expressed on weight) were measured for each pair of single
366 host evolutionary histories in the two corresponding hosts and we could perform the classical
367 test for local adaptation (Kawecki, Ebert 2004). For each of the six pairs of single-host
368 evolutionary histories and for each host, the difference between the trait value of the local (or
369 native) evolutionary history and that of the foreign (or non-native) evolutionary history was
370 calculated. If this difference is positive, it indicates local adaptation, whereas if it is negative,
371 it indicates local maladaptation. This analysis assumes that our variables are positively related
372 to fitness, which is the case for infectivity and is arguably the case for virulence (see
373 Introduction and Discussion). For each variable, a distribution of twelve values was obtained
374 and it was possible to test whether the differences were on average positive. The mean is

positive for the three variables but not significantly different from zero for any of them: infectivity (1-tailed *t*-test: $p = 0.096$; Wilcoxon rank test: $p = 0.071$), virulence expressed on size (1-tailed *t*-test: $p = 0.069$; Wilcoxon rank test: $p = 0.17$) and virulence expressed on weight (1-tailed *t*-test: $p = 0.064$; Wilcoxon rank test: $p = 0.073$). However, the combined Fisher test reveals an overall significant local adaptation (combining 1-tailed *t*-tests: $p = 0.017$; combining rank tests: $p = 0.029$). Then, using the same differences, it is possible to evaluate the frequency at which the adaptation in one host comes to a cost of adaptation in the other host. For the six pairs of single host evolutionary histories, we compared the differences previously calculated in each of the two hosts. For example, we compared the (local – foreign) difference in infectivity of the Nb and Nt lineages when inoculated on *N. benthamiana* with their difference in infectivity when inoculated on *N. tabacum*. If the two differences are positive, then we have a case of local adaptation with a cost of adaptation. Out of the 18 cases (six pairs × three variables), there are seven cases of local adaptation with a cost of adaptation, ten cases of local adaptation of one of the evolutionary histories without cost expressed in the other host and one case of double maladaptation. Out of the ten cases of adaptation without cost, five concern lineages evolved on *D. stramonium*: the adaptation to *D. stramonium* seems thus to be compatible with the infection and virulence expression in other hosts. Finally, for three of the six pairs of single host evolutionary histories, data are available for the corresponding alternate host evolutionary histories. Out of the seven cases where a cost of adaptation has been identified, we have data for the corresponding alternate host evolutionary history in four cases. There is no clear cost of being a generalist (which would be expressed as a lower value for the generalist strategy than for the locally adapted specialist strategy in each host) in any of these four cases, confirming the idea that the specialist-generalist trade-off is not present in this experimental system. Figure 4 represents the data for virulence expressed on weight for the (Nt, Ca, NtCa) group of evolutionary histories, as an

example of a situation with local adaptation, a cost of adaptation and no cost for the corresponding generalist.

Consensus sequences

A total of 107 independent mutations occurring at 91 different loci were identified with a range of zero to six mutations per independent evolved lineage in the 69 independently evolved lineages (see figure 5 for a graphical representation and table S1 for a complete list). The transition:transversion ratio was 7.2. Sixty-two mutations were synonymous and 45 non-synonymous, the biological interpretation hereof is discussed below. Out of the 107 mutations observed, 26 (24%) were not unique and out of the 91 polymorphic loci identified, 10 (11%) were affected in multiple independent lineages. All the non-unique mutations were repeated in lineages that shared a host in their evolutionary history, except in one case (A5409G present in one Nb and one NtCa lines). The very large majority (24 out of 26) of non-unique mutations were thus specific to one of the hosts used in the experiment.

We then tested whether the regions encoding for each of the eleven mature proteins had a different rate of mutation accumulation and whether this depended on the evolutionary history of the lineage. To do this, we calculated the number of mutations per nucleotide site for each evolutionary history and for each mature protein. A Scheirer-Ray-Hare nonparametric two-way ANOVA (Sokal, Rohlf 1995) showed that the mutation accumulation per nucleotide is not globally different from one evolutionary history to another, but that mutations are not randomly scattered across mature proteins and that this distribution is different from one evolutionary history to another (Table 5). Namely, there is an overrepresentation of mutations in P1 in lineages Ca, Nt and NtCa, of mutations in HC-Pro in lineages Ds and DsCa, of mutations in P3 in lineages Nb and NbNt, of mutations in CI in lineages NtCa, of mutations in 6K2 in lineages Nb, of mutations in VPg in lineages Ds and of mutations in NIa-Pro in lineages Ca.

Focussing on individual consensus sequences, it is possible to identify patterns of mutations that suggest a potential relationship between genotype and phenotype. First, among the Nb lineages, there are two mutations repeated three times, and there is no case where both are present in the same consensus sequence. Moreover, one of them (A3013G, non-synonymous K→E) was also found in three independent NbNt lineages whereas the other one (A5551G, non-synonymous K→E) was not found in any of the NbNt sequence. This suggests that there are at least two mutations that can improve the adaptation to infection and reproduction in *N. benthamiana* and that one (A3013G) is neutral or advantageous whereas the other one (A5551G) is deleterious in *N. tabacum*. Second, an identical combination of four mutations (A3013G, C3816A, A6805G, and G8169A) has been found in lineages Nb8 and NbNt6. Except the mutation A3013G, the other three have not been found in any other lineage besides these two. This could be due to the existence of epistatic interactions in this group of mutations. Third, lineages Ca1 and Ca2 have lost the ability to produce systemic infections when inoculated on *N. tabacum* and on *D. stramonium* and form only local lesions on the inoculated leaf, but still produce systemic symptoms on *C. annuum* and *N. benthamiana*. The analysis of their sequence revealed only one synonymous mutation in Ca1 (C4425U in the CI region) and two synonymous mutations in Ca2 (A183G and U8574C in the P1 and CP region, respectively).

The relationship between the mutations and the phenotype observed are clearly correlational and speculative for now and the evolutionary mechanisms suggested by the analysis of the sequences have to and will be directly tested in future site-directed mutagenesis experiments.

Discussion

TEV evolved differently when experimentally exposed to different evolutionary histories, as evidenced by the phenotypic traits measured, as well as by the sequence changes observed in the evolved lineages. The ancestral virus and the lineages evolved on *N. tabacum*, the ancestral host, present different patterns of infectivity and virulence. This is likely due to an adaptation to the specific conditions of the experimental evolution and in particular to the mechanical transmission, which totally removes the selection due to the aphid transmission. The transmission mode is actually known to affect viral evolution both at the phenotypic and at the genotypic levels (Wallis et al. 2007; Jerzak et al. 2008). Due to these differences between the ancestor and the Nt lineages, we focused the analysis and the discussion on the comparison of the experimental evolutionary histories between them.

We observe that the viral populations have adapted to the hosts that they encountered during their specific evolutionary histories. The analyses performed on pairs of populations further confirm the pattern of local adaptation. In some systems, local adaptation comes at a cost (Hereford 2009), expressed as a worse performance of the locally adapted populations when measured in a foreign environment. In the case of TEV on the four hosts used, the cost of adaptation is not a general feature, as it appears only in some cases. The results for infectivity and virulence were congruent in direction, albeit not in magnitude for all the analyses performed.

The consensus sequences of the evolved populations revealed common mutations between lineages sharing host species in their evolutionary histories. Such parallel evolution is usually interpreted as the fixation of a mutation with a beneficial effect (Wood, Burke, Rieseberg 2005), and in this precise case, the host-specific mutations likely increase the level of adaptation to the particular host species. One potential concern about the experimental protocol is that the removal of the aphid vector from the virus life-cycle would relax selection

474 pressures at some loci, e.g. in the N-terminal part of HC-Pro known to be involved in aphid
475 transmission (Blanc et al. 1998) and that part of the mutations observed would be due to this.
476 The absence of common mutations between lineages that do not share a host excludes the
477 relaxed selection pressure of aphid transmission as the main evolutionary force shaping
478 sequence during our evolution experiment.
479 Overall, the level of parallel evolution obtained here is below those in other studies
480 comparing genomes from lineages sharing evolutionary history (e.g., Bull et al. 1997;
481 Martínez-Picado et al. 2000; Wichman, Millstein, Bull 2005; Remold, Rambaut, Turner
482 2008). In particular, the experiment conducted by Remold et al. (2008) presents a number of
483 similarities with the present one: it involves experimental evolution of a RNA virus (VSV) by
484 serial transfers either in one of two cell cultures (human or hamster cells) or in the alternation
485 of the two cell types, followed by full genome sequencing of twelve evolved populations. The
486 authors found that 78% of the observed mutations were not unique and 55% of the
487 polymorphic loci were affected in multiple independent populations. The reasons for the
488 lower level of parallel evolution in our system are likely manifold. First, this level strongly
489 depends on the genetic architecture of adaptation, *i.e.* on the distribution of mutation effects in
490 the organisms and in the environments considered (Chevin, Martin, Lenormand 2010) but, the
491 shape of these distributions is rarely known and consequently the role of this factor in
492 determining the level of parallel evolution cannot be evaluated on concrete evolution
493 experiments. Second, the lower level of parallel evolution in our study could be due to the
494 narrower and less controlled bottlenecks between passages: we controlled the amount of viral
495 RNA inoculated but the number of virions actually starting the new infection can vary
496 considerably (Zwart, Daròs, Elena 2011). In any case, the number of virions starting a new
497 infection represents an extremely small fraction of those produced during the colonisation of
498 the host in the previous passage and a much smaller fraction than in the case of serial transfer

of virus in cell culture. This reduction in effective population size increases the role of genetic drift and reduces the likelihood of fixation of the same mutation in two replicates of the same evolutionary treatment. Third, populations of plant viruses are highly structured because (i) infection within a leaf progresses only from one cell to adjacent ones, (ii) viral exclusion phenomenon are frequent (Dietrich, Maiss 2003; Zwart, Daròs, Elena 2011) and reduce the generation of diversity by recombination, and (iii) the order of colonization of the leaves is fixed by the phloem distribution. All these factors generate much more opportunities for local competition and exclusion of alleles, more heterogeneity in selection pressures and result in a much smaller effective population size than a well-mixed cell culture. Finally, the low level of parallel evolution might come from the fact that we performed a passage every week during experimental evolution. One week might represent an insufficient time for the virus to generate genetic diversity and for the selection to act on this diversity. However, first for technical reasons and second owing to the drastic effect of TEV on some of the hosts used in this experiment (see virulence levels on *N. benthamiana*), it was unrealistic to perform longer passages.

For the analysis of consensus sequences, we decided not to interpret the synonymous/nonsynonymous characteristic of the mutations as neutral/non-neutral. Traditionally synonymous mutations have been considered neutral because they do not induce amino acid changes and their proportion relative to the nonsynonymous ones has been used to deduce the evolutionary forces at work. However, there is accumulating evidence that synonymous mutations are not always neutral in DNA organisms (Kimchi-Sarfaty et al. 2007; Amorós-Moya et al. 2010; Plotkin, Kudla 2011) and the equivalence of synonymous with neutral is likely to be even weaker in RNA viruses, because tridimensional structure is a determinant feature of RNA molecules and it is largely dependent on the sequence itself. The RNA-based genome can thus itself potentially be a target of selection, because coding regions

524 serve additional functions other than determining the amino acid sequence in protein. These
525 additional functions include encapsidation or serving as a target of silencing (Cuevas,
526 Domingo-Calap, Sanjuán 2011). In TEV, it has been shown that certain synonymous
527 mutations had deleterious effect on the virus multiplication (Carrasco, de la Iglesia, Elena
528 2007) and the present study provides a striking example of non-neutrality of synonymous
529 mutations with the two lineages which lost the capacity to produce systemic infection on
530 certain hosts and only carry synomymous mutations.

531 Another interesting result of our study is the absence of cost of generalism, either
532 when we look at the full data set or when we can contrast the characteristics of one generalist
533 evolutionary history with the ones of the two corresponding specialists. This is probably
534 partly due to the fact that the cost of adaptation is not general and consequently, that the
535 adaptation to two hosts simultaneously does not represent a strong evolutionary constraint.
536 However, what might seem harder to explain is the pattern of reciprocal local adaptation with
537 a cost of adaptation and without any identified cost of generalism (e.g. figure 4). A pattern of
538 reciprocal local adaptation with a cost of adaptation can be due either to antagonistic
539 pleiotropy or to fixation of mutations beneficial in the local host and neutral in the other one.
540 Indeed, in potyviruses, we can exclude the accumulation of deleterious mutations at loci not
541 expressed in one of the hosts (Kawecki 1994) as the full genome is constantly expressed as a
542 single polyprotein. If the cost of adaptation was due to antagonistic pleiotropy, the generalists
543 should pay a cost. Antagonistic pleiotropy makes impossible the adaptation to two hosts at the
544 same time. If it was due to neutral mutations, they could perform as well as the best lineage in
545 each host. Our experimental results favour the second explanation. However, the previous
546 reasoning is based on phenotypic traits only and the sequence data indicate a low level of
547 parallel evolution, meaning that the adaptation is due to different mutations in replicate
548 lineages of an evolutionary history and between evolutionary histories sharing a host. This

549 weakens considerably the argumentation based on phenotypic traits and knowing really
550 whether the mutations obtained are antagonistically pleiotropic would require analysing them
551 one by one through site-directed mutagenesis of the wild-type TEV genome. Finally, another
552 potential, non-exclusive explanation for the absence of cost of generalism is that the
553 "generalist populations" could actually be a composite of two populations, each one being
554 adapted to one of the hosts. If this was the case, it should be visible on the consensus
555 sequence data with a highest proportion of identified polymorphic sites in generalist than in
556 specialist populations. This is however not the case: polymorphic sites represent 24.4% of the
557 sites where a change has been identified in generalist lineages compared to 28.8% in
558 specialist lineages (Fisher's exact test, $p = 0.763$).

559 To sum up, our experimental evolution of TEV in a multihost context revealed a
560 pattern of local adaptation, characterized in particular by a higher infectivity on the host(s)
561 encountered during the experimental evolution. Local adaptation did not always come with a
562 cost of adaptation and the cost of generalists predicted by theoretical approaches was not
563 found. At the sequence level, host specific mutations were found but the general level of
564 parallel evolution was relatively low. Altogether, these experimental results reveal a strong
565 adaptation potential of TEV to new hosts without severe evolutionary constraints. Our
566 experiment, using an *in vivo* system and a larger replication of and within evolutionary
567 histories, points in the same direction as previous experimental evolution approaches on other
568 pathogens. The generality of the high potential of adaptation of pathogens to hosts, which are
569 new to them at the evolutionary scale considered, indicates that emerging diseases caused by
570 a diversity of organisms actually represents an important threat. The most efficient policies to
571 limit them are preventive and should target the steps prior to adaptation: limit the flow of
572 pathogens towards new host species and avoid generating evolutionary situations favourable
573 to adaptation to a new host.

574

575 **Supplementary material:** see supplementary files. Contains supplementary methods,
576 supplementary table 1 and supplementary figure 1.

577

578 **Acknowledgement:** We thank Francisca de la Iglesia and Àngels Pròsper for
579 excellent technical assistance and Mark Zwart and two anonymous reviewers for their helpful
580 comments on a previous version of the manuscript. This research was supported by the
581 Spanish Ministry of Science and Innovation grant BFU2009-06993 to SFE. SB was supported
582 by the JAE-doc program from CSIC and GL was supported by the Human Frontier Science
583 Program, grant RGP0012/2008.

584

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743 **Tables.**

744 *Table 1.* Nominal logistic model on the infectivity data of the experimentally evolved

745 lineages.

746

Factor	*d.f.*	*Likelihood ratio* χ^2	*p*
Evolutionary history	6	115.92	<0.0001
Host	3	166.34	<0.0001
Evol. hist. × host	18	83.01	<0.0001
replication [evol. hist.]	46	158.77	<0.0001

747

748

749 *Table 2.* ANOVA of the virulence data (expressed on size and on weight) of the

750 experimentally evolved lineages.

751

Virulence expressed on size

Factor	*d.f. denominator*	*d.f. numerator*	*F ratio*	*p*
Evolutionary history	6	49.66	2.06	0.0752
Host	3	311.34	215.99	<0.0001
Evol. hist. × host	18	304.81	3.95	<0.0001
replication [evol. hist.] (Rdm)		*percentage of variance explained*		23.61%

Virulence expressed on weight

Factor	*d.f. denominator*	*d.f. numerator*	*F ratio*	*p*
Evolutionary history	6	43.23	0.805	0.5713
Host	3	317.78	10.937	<0.0001
Evol. hist. × host	18	311.51	1.843	0.0202
replication [evol. hist.] (Rdm)		*percentage of variance explained*		9.51%

752

753

754 *Table 3*. Effect of the type of evolutionary history (specialist or generalist) and of the type of

755 infection (foreign or local) on infectivity.

756

Factor	*d.f.*	*Likelihood ratio* χ^2	*p*
Host	3	150.52	<0.0001
Foreign/Local	1	7.58	0.0059
Specialist/Generalist	1	0.41	0.5222
Host × F/L	3	24.35	<0.0001
Host × S/G	3	2.04	0.5635
F/L × S/G	1	0.71	0.4008
Host × F/L × S/G	3	2.53	0.4704

757

758

759 *Table 4.* Effect of the type of evolutionary history (specialist or generalist) and of the type of

760 infection (foreign or local) on virulence.

761

Virulence expressed on size			
Factor	*d.f.*	*F ratio*	*p*
Host	3	255.14	<0.0001
Foreign/Local	1	15.05	0.0001
Specialist/Generalist	1	0.03	0.8655
Host × F/L	3	5.62	0.0009
Host × S/G	3	1.20	0.3100
F/L × S/G	1	2.81	0.0943
Host × F/L × S/G	3	3.32	0.0199
Virulence expressed on weight			
Factor	*d.f.*	*F ratio*	*p*
Host	3	17.590	<0.0001
Foreign/Local	1	6.460	0.0114
Specialist/Generalist	1	0.365	0.5459
Host × F/L	3	1.791	0.1482
Host × S/G	3	0.782	0.5043
F/L × S/G	1	0.727	0.3943
Host × F/L × S/G	3	1.009	0.3885

762

763

764 *Table 5.* Nonparametric analysis of variance for the number of nucleotide substitutions among

765 genes and for each experimental evolutionary history.

766

Term	*d.f.*	*H*	*P value*
Evolutionary history	6	10.81	0.0944
Genome region	9	30.80	0.0003
Evol. hist. × Gen. region	54	103.57	<0.0001

767

768

Figure legends

Figure 1. Overview of the experimental evolution procedure. The seven evolutionary histories are represented: in continuous lines, the "specialist" history and in dotted lines, the evolutionary histories with host alternation. Host species are codified as follows: Ds = *D. stramonium*, Ca = *C. annuum*, Nt = *N. tabacum* and Nb = *N. benthamiana*.

Figure 2. Infectivity of the different evolutionary histories on the four experimental hosts. Infectivity is expressed in terms of proportion of plants infected ± 1 SEM.

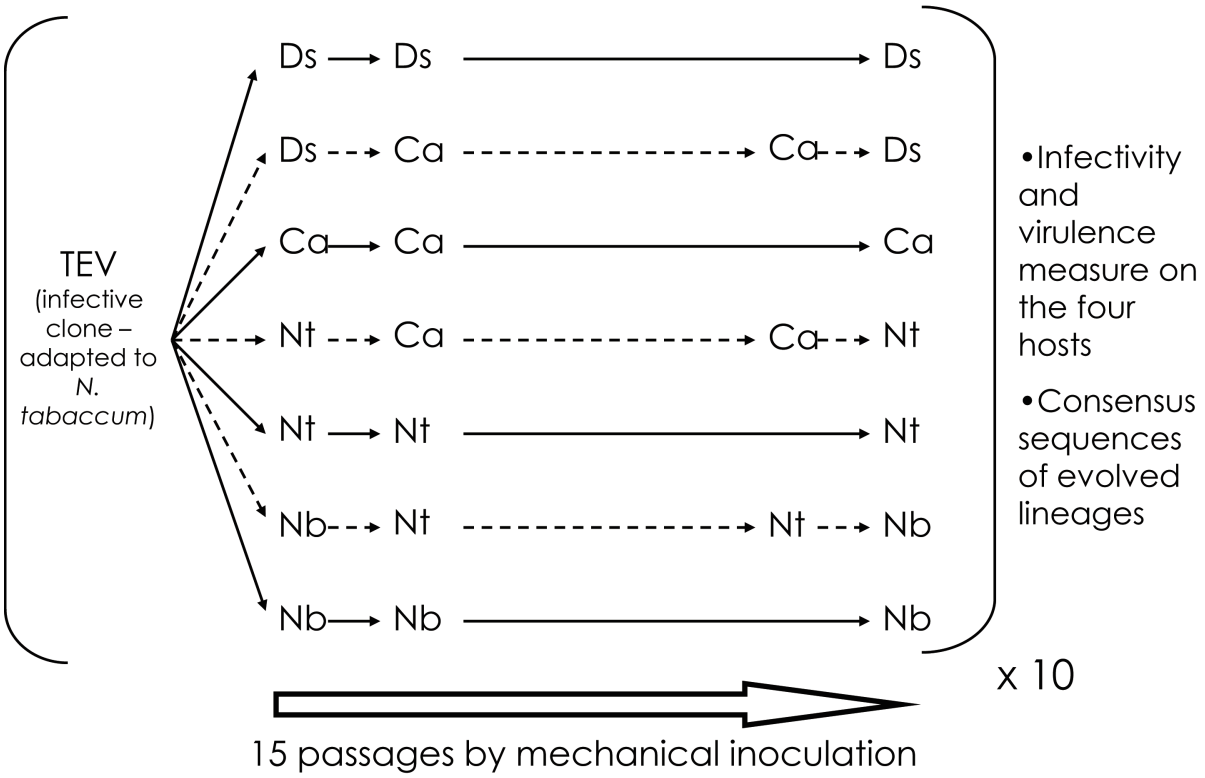
Figure 3. Experimentally evolved local adaptation: infectivity (A), virulence expressed on size (B) and virulence expressed on weight (C) of local (triangles) and foreign (circles) across the four experimental hosts. All values are expressed ± 1 SEM.

Figure 4. Virulence expressed on weight (± 1 SEM) for the Nt (triangles), Ca (circles) and NtCa (squares) lineages, when measured on *C. annuum* and *N. tabacum*.

Figure 5. Schematic representation of the collection of mutations obtained in the 69 experimentally evolved lineages. The first line represents the full TEV genome with the position of the eleven mature viral proteins within the ORF. The seven other lines correspond each to one evolutionary history and all the mutations obtained for this evolutionary history are represented. Synonymous mutations are represented as empty circles and non-synonymous mutations are represented as full circles. Mutations in black are unique whereas mutations in colour are shared between several lineages. The colour corresponds to the host present during the evolutionary history of all the lineages containing the shared mutation and

794 the colour code is reported on the evolutionary history names on the left. The number in
795 parentheses above each shared mutation indicates the number of times the mutation has been
796 found in the evolutionary history.
797

798 Figure 1.



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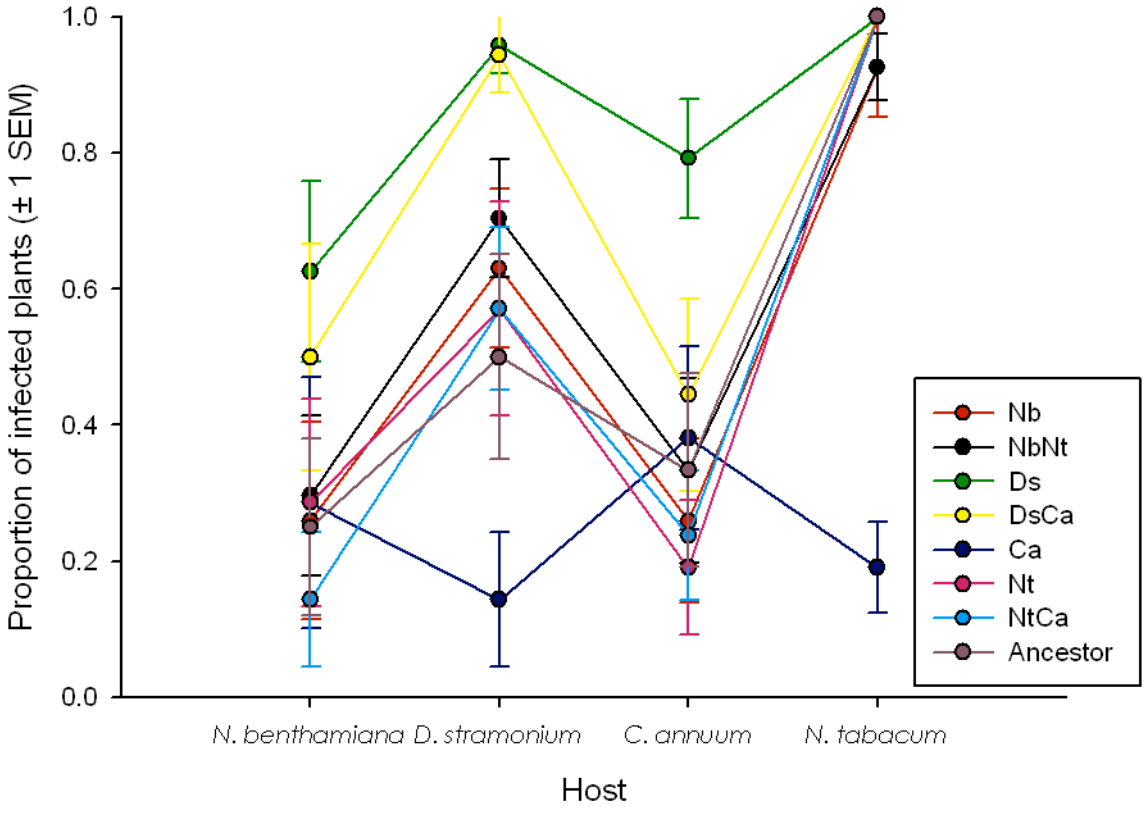
801 Figure 2



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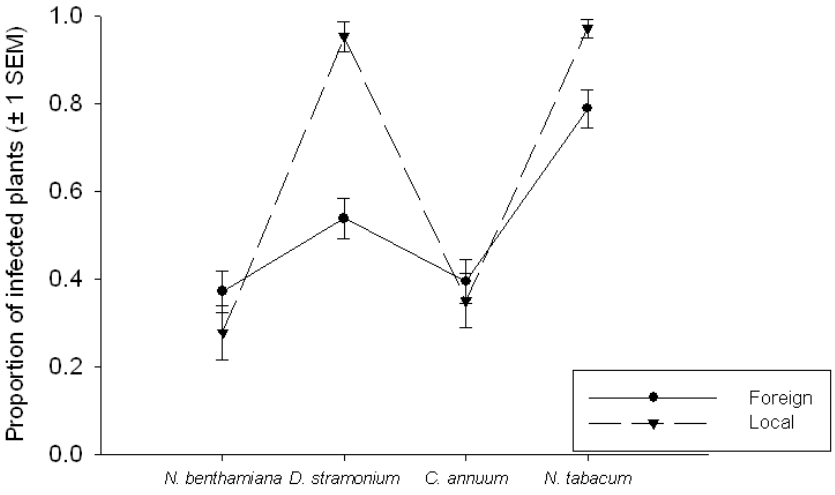
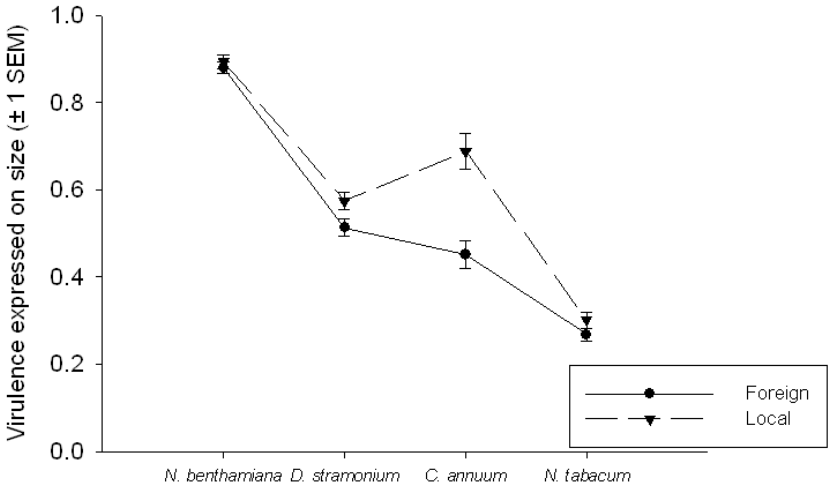
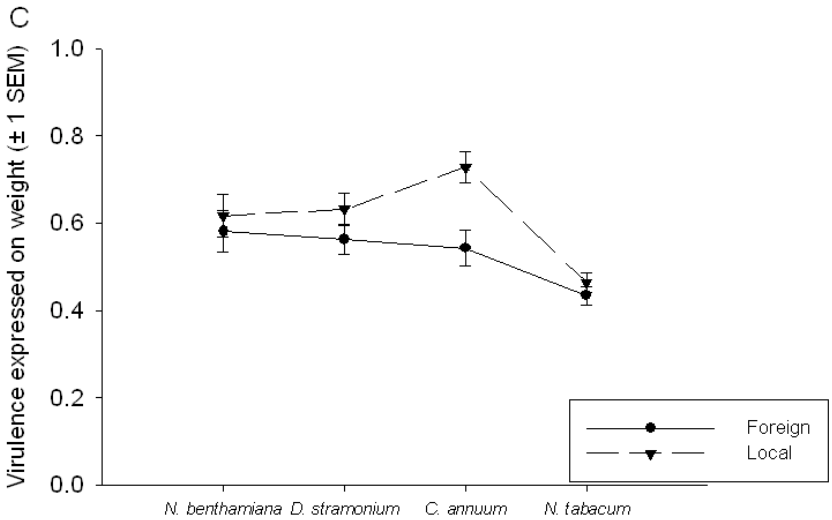
804 Figure 3







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807 Figure 4

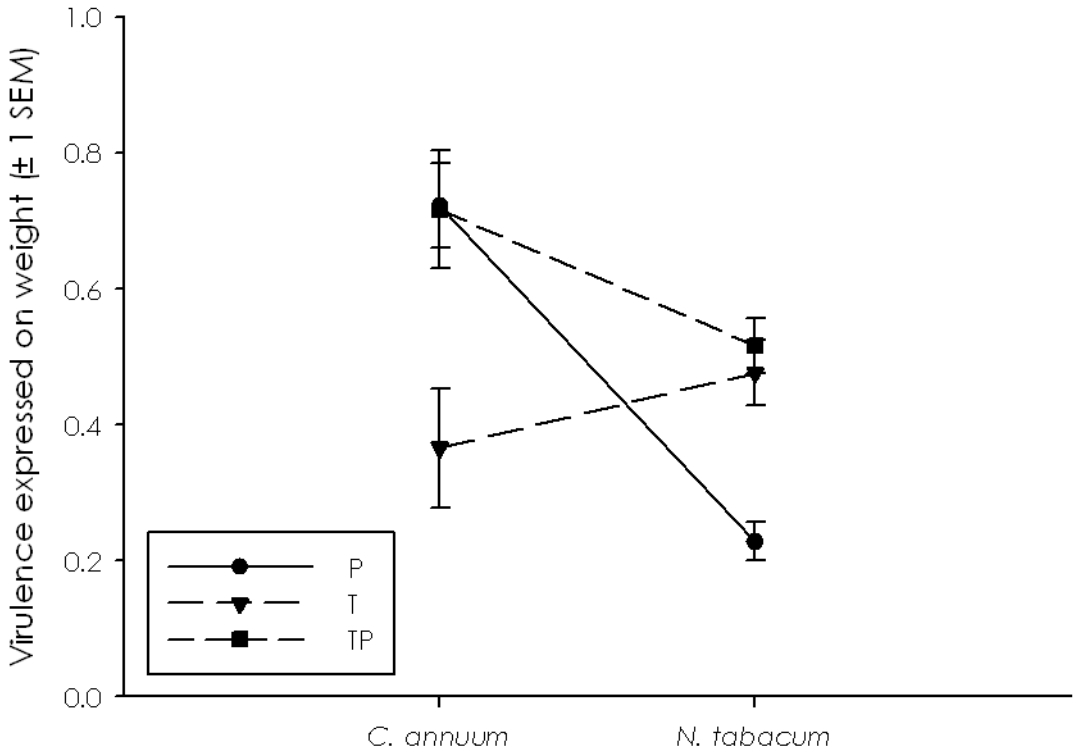

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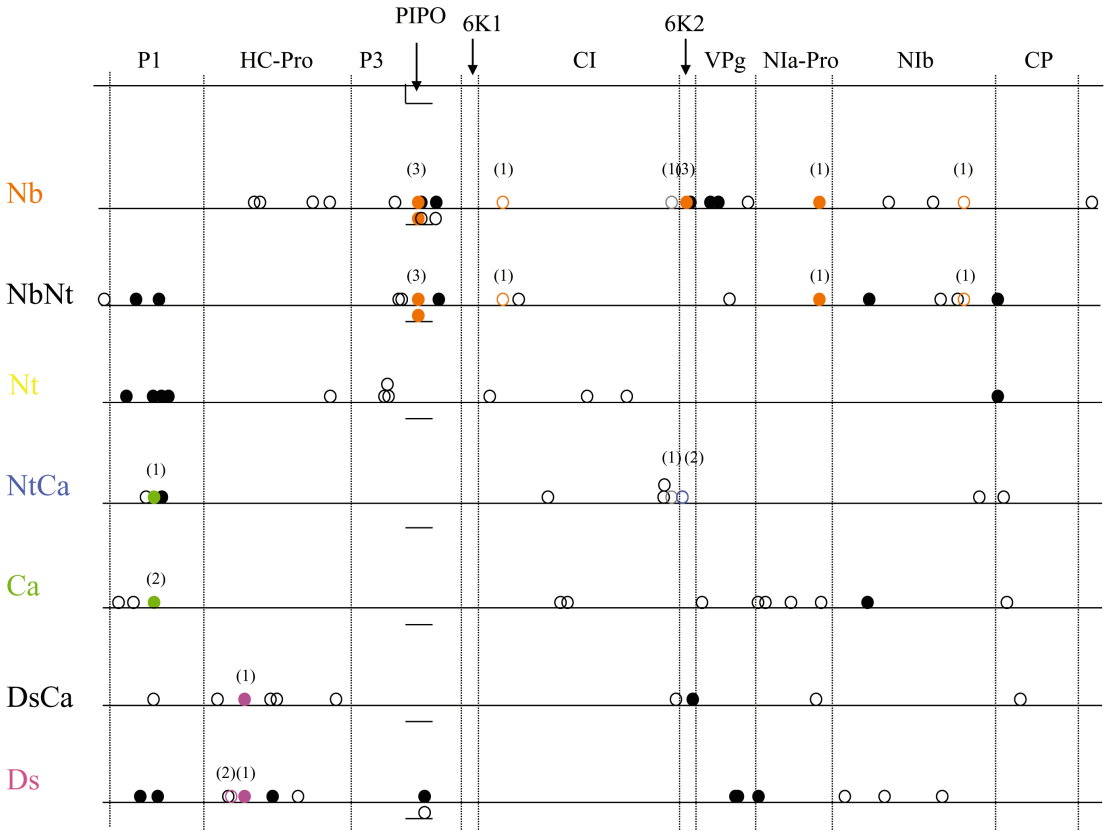
Figure 5.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6K2
P1
HC-Pro
P3
CI
VPg
NIa-Pro
NIb
CP
Nb
NbNt
Nt
NtCa
Ca
DsCa
Ds
(3)
(1)
(1)(3)
(1)
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(2)
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