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Review Article

Reassortments in single-stranded DNA multipartite viruses: Confronting expectations based on molecular constraints with field observations

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Abstract

Single-stranded DNA multipartite viruses, which mostly consist of members of the genus *Begomovirus*, family *Geminiviridae*, and all members of the family *Nanoviridae*, partly resolve the cost of genomic integrity maintenance through two remarkable capacities. They are able to systemically infect a host even when their genomic segments are not together in the same host cell, and these segments can be separately transmitted by insect vectors from host to host. These capacities potentially allow such viruses to reassort at a much larger spatial scale, since reassortants could arise from parental genotypes that do not co-infect the same cell or even the same host. To assess the limitations affecting reassortment and their implications in genome integrity maintenance, the objective of this review is to identify putative molecular constraints influencing reassorted segments throughout the infection cycle and to confront expectations based on these constraints with empirical observations. Trans-replication of the reassorted segments emerges as the major constraint, while encapsidation, viral movement, and transmission compatibilities appear more permissive. Confronting the available molecular data and the resulting predictions on reassortments to field population surveys reveals notable discrepancies, particularly a surprising rarity of interspecific natural reassortments within the *Nanoviridae* family. These apparent discrepancies unveil important knowledge gaps in the biology of ssDNA multipartite viruses and call for further investigation on the role of reassortment in their biology.

Keywords: reassortment; multipartite viruses; ssDNA viruses; *Nanoviridae*; *Geminiviridae*; *Begomovirus*.

General introduction

Depending on their genomic organization and packaging strategy, viruses can be monopartite, segmented, or multipartite. While monopartite viruses encode all their genetic information in a single nucleic acid molecule packaged within a viral particle, the other two categories have fragmented genomes but package their segments either all together in the same (segmented) or in separate particles (multipartite). The physical separation of the genetic information in segmented and multipartite viruses allows the replacement of entire segments by homologous ones from a distinct parental genotype through a genetical exchange mechanism known as reassortment or pseudo-recombination (Roossinck 1997; Holland and Domingo 1998; McDonald et al. 2016). Like recombination, reassortment may disrupt co-adapted gene complexes by generating hybrid genotypes (Martin et al. 2011a, 2011b; Varsani et al. 2018), but in return, it can also promote genetic innovation (Roossinck 1997; Holland and Domingo 1998; Martin et al. 2011b), potentially playing an important role in evolution and adaptation (Chao, Tran, and Matthews 1992; Martin et al. 2011b).

While reassortant genotypes may occur frequently (Matsuzaki et al. 2003) and can be associated with substantial alterations in viral traits, such as hypervirulence (Chakraborty et al. 2008), resistance-breaking (Kwon et al. 2021), or host range expansion (Idris et al. 2008), reassortments are also often associated with deleterious effects (Escriu, Fraile, and García-Arenal 2007; Ohshima et al. 2016; Villa and Lässig 2017). Overall, we presently lack a systematic characterization of the phenotypic effects of reassortments, such that their net effect on viral fitness is unknown for any viral taxon.

Although not duly emphasized in the literature (but see Varsani et al. 2018), segmented and multipartite viruses differ in their potential to undergo reassortment. In principle, for segmented viruses, reassortment implies co-packaging of

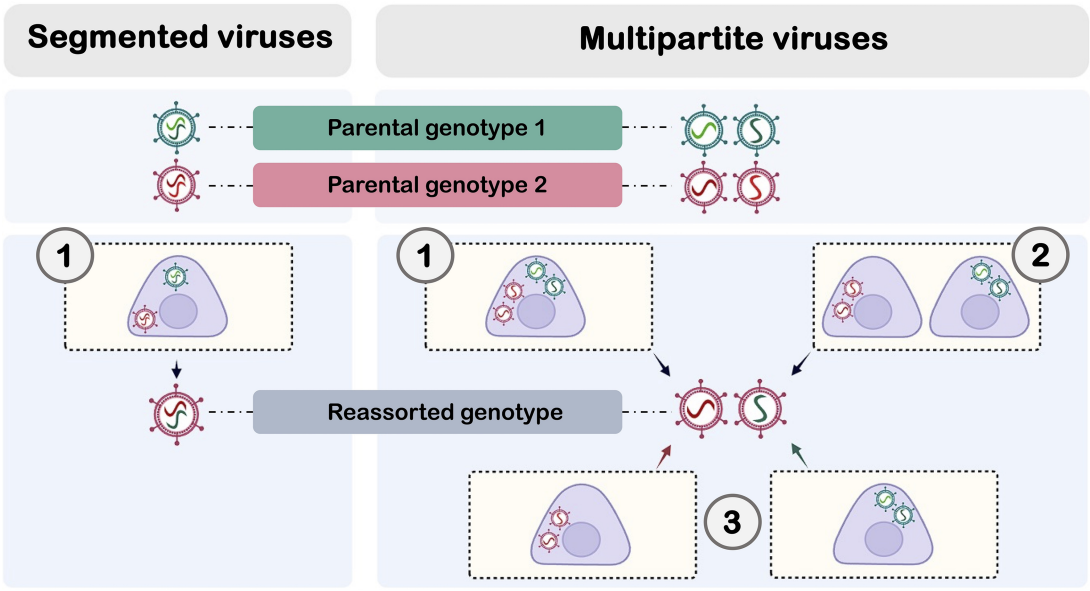

Figure 1. Comparison of the reassortant production processes in multipartite and segmented viruses. (1) Two viral genotypes co-infect the same cell. A reassortment results from the co-packaging of two segments, each originating from a distinct parental genotype in the case of segmented viruses and from the production of a population of particles containing complementary segments from two parental genotypes for multipartite viruses. (2) Two viral genotypes co-infect the same host. Some ssDNA multipartite viruses follow a multicellular infection cycle where functional complementation occurs at a supra-cellular level. When a host is co-infected by two parental genotypes, their distinct segments can infect separate cells and interact through complementation, which can result in the formation of a reassorted genotype. (3) Two viral genotypes infect different hosts. Reconstitution of a reassorted genome can result from the separate transmission of two particles containing complementary genomic segments originating from distinct host and parental genotypes. Triangles represent individual cells; rectangles represent host individuals. Parental and reassorted genotypes are indicated for both segmented and multipartite viruses. Distinct segments of a given genotype are colored and oriented differently.

heterologous segments, while in multipartite viruses, distinct segments are packaged separately (Fig. 1). Recent discoveries suggest that this fundamental difference may have far-reaching implications. A study showed that the single-stranded DNA (ssDNA) multipartite faba bean necrotic stunt virus (FBNSV, *Nanovirus* genus) exhibits a unique 'pluricellular lifestyle' (Sicard et al. 2019). Using sequence specific fluorescence in situ hybridization, the authors demonstrated that the different genomic segments rarely co-occur in the same cell, the viral system functioning through complementation across multiple cells (Sicard et al. 2019). A subsequent study showed that it was possible to reconstitute a complete genome from complementary incomplete segment sets infecting distinct host plants (Di Mattia et al. 2022). To achieve such complementation, recipient plants could be inoculated by distinct aphid vector individuals, each carrying a complementary set of genome segments, or inoculated by a single aphid that sequentially acquired the two complementary segment sets (Di Mattia et al. 2022). The capacity to transmit genomic segments separately between cells and even between hosts implies that, contrary to segmented viruses, at least nanoviruses do not require to co-infect the same cell or even the same host to exchange segments, drastically increasing their potential to reassort (Fig. 1).

Reassortments may thus play a prominent role in the life cycle of these viruses, if the conditions to reassort are met. A favorable ecological context is necessary, which involves parental genotypes sharing host or vector species within the same geographical area. As a result, reassortments tend to occur at geographical hotspots where multiple species or isolates co-exist (Savory, Varma, and Ramakrishnan 2014; Xavier et al. 2021). In the presence of such diversity, the chances of genome reconstitution would further increase if viable reassortants were frequent. However, reassortments may be subject to multiple molecular constraints. Not only the reassorted segments have to functionally complement their new genomic background, but they must also be complemented by it as they undergo replication, packaging, intra- and inter-host movement to generate an infection focus (Fig. 2).

Given the potential importance of genome integrity maintenance through genome reconstitution demonstrated in nanovirids and the associated significance of reassortant viability, we here review the potential ecological and molecular constraints affecting reassortments in ssDNA multipartite viruses. This literature survey generates predictions based on laboratory experiments on the stages of the viral life cycle most likely to affect reassortant viability, which are subsequently confronted to the available data on the properties and frequency of reassortants artificially produced or found in field samples. We here assume, awaiting further demonstration, that the property for genomic segments to propagate separately within and between hosts is not unique to nanoviruses, but may in fact be shared by at least other ssDNA multipartite viruses. We thus expand our survey and arguments on other such viruses and primarily on the extensively studied multipartite geminivirus species.

Geminiviridae—*Begomovirus*

Introduction

Until recently, the only members of the family with a non-monopartite genome belonged to the genus *Begomovirus*. The Olea europaea geminivirus (OEGV) is currently unclassified but is likely to represent its own genus within the *Geminiviridae* family (Materatski et al. 2021). OEGV is bipartite and shares similarities in genome size and DNA-A organization with bipartite begomoviruses, but it differs in its DNA-B organization and overall

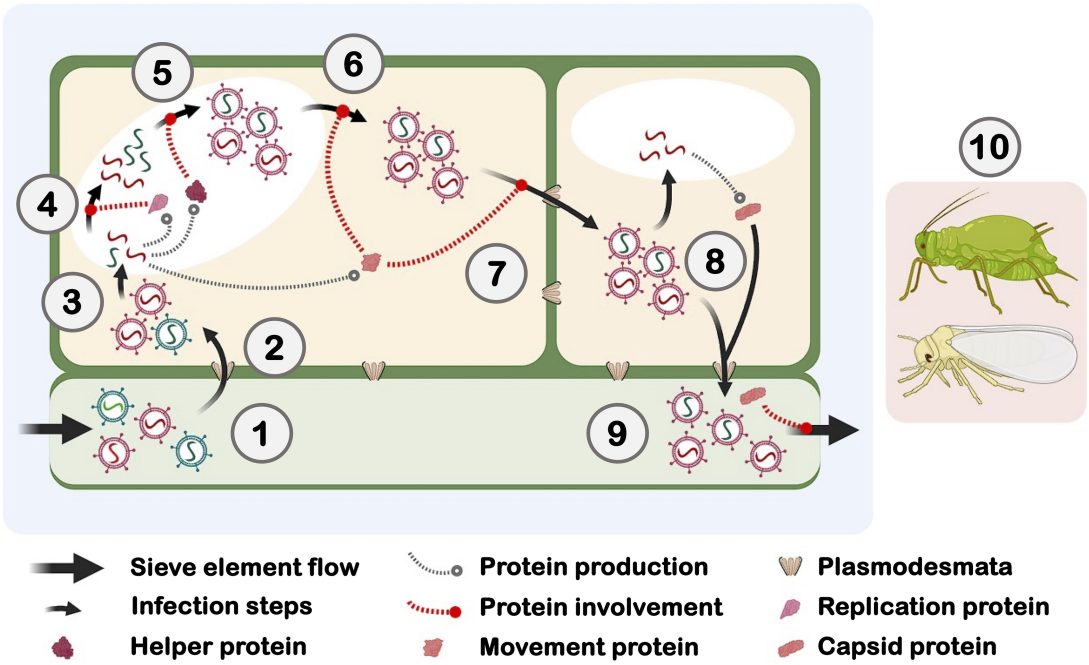

Figure 2. Overview of the infection cycle key steps where molecular constraints may impact segment compatibility upon reassortment of multipartite viruses. (1) This scenario illustrates a host plant co-infected by two bipartite virus genotypes (green and red) first released into the sieve element. (2) All types of viral particles can invade companion cells through plasmodesmata. (3) Decapsidation enables viral DNA to replicate within the nucleus and leads to the transcription and translation of associated proteins. (4) A reassortant is produced when, e.g. the replication protein of the green genotype can replicate the complementary segment of the red genotype, which can then be successfully encapsidated and transmitted, or *vice versa*. (5) Reassorted viral DNA is packaged by the CP. There, reassortment might be facilitated by the capacity of the CP to package heterologous ssDNA. (6/7/8) Viral particles leave the nucleus thanks to the intra- and intercellular MPs allowing any ssDNA segment to invade neighboring cells. (9) Virions travel long distance in the vasculature. The movement and CPs can be involved for this long-distance progression, and thus, compatibility between them may be required. (10) Insect vectors acquire viral particles while feeding on the host plant. Reassortant transmission depends on the compatibility between the vector species, the CP, and a potential transmission helper protein. Distinct segments of a given genotype are colored and oriented differently.

nucleotide identity. Specifically, its movement protein (MP) is in the virion sense, while a small undetermined open reading frame (ORF) is in the complementary sense (Chiumenti et al. 2021). Limited information is available about this virus, except that it appears to be highly prevalent in olive trees, and its isolates show a very close sequence conservation (99 per cent pairwise identity) (Alabi et al. 2021), making it difficult to detect reassortment events. Therefore, in this section, we will focus on begomoviruses.

The largest and most extensively studied group of ssDNA plant viruses is the genus *Begomovirus* comprising both monopartite and bipartite species. Begomoviruses are a significant threat to a wide range of crops, especially in subtropical and tropical regions, and are thus the object of many studies.

Bipartite begomoviruses have a genome consisting of two circular segments named DNA-A and DNA-B, each of approximately 2.7 kb (Rojas et al. 2005) (Fig. 3). DNA-A commonly has six ORF: in the virion sense, AV1 encoding the only structural protein (capsid protein (CP)) and AV2 encoding the pre-coat protein (PCP), involved in movement and gene silencing, that is absent in some species, and in the complementary sense, AC1 encoding the replication-associated protein (Rep), AC2 encoding a transcriptional activator protein (TrAP), AC3 encoding a replication enhancer (REn), and AC4 encoding (RepA) also involved in the replication process. DNA-B carries two ORFs: in the viral sense, BV1 encoding a nuclear shuttle protein (NSP) and in the complementary sense, BC1 encoding a MP. Recent studies identified new candidate ORFs with putative homologs in bipartite species (Gong et al. 2021; Chiu et al. 2022).

Begomoviruses are often associated with alpha-, beta-, or delta- satellites, which are facultative subviral agents relying on their helper virus for essential viral functions such as replication, encapsidation, and transmission. Delta- and beta-satellites are dependent on their helper virus to replicate (Mubin et al. 2020; Ferro et al. 2021), whereas alpha-satellites are responsible for their own replication and do not replicate the helper's segments (Mansoor et al. 1999; Briddon et al. 2018). Satellites can form associations with new helper species following reassortments (Chen et al. 2009; Mubin et al. 2020). The impact of satellites on their helper viruses is highly heterogeneous and can be beneficial in some instances. For example, some beta-satellites have been described to substitute for a DNA-B in bipartite species or to increase the ability of their helper virus to infect a different host plant species (Saeed et al. 2007; Singh, Chattopadhyay, and Chakraborty 2012; Mubin et al. 2020).

Most begomoviruses are phloem-restricted, but a few bipartite species can also infect mesophyll tissues (Morra and Petty 2000). They are all transmitted by whiteflies, most notably of the complex of cryptic species *Bemisia tabaci* (Gilbertson et al. 2015; Fiallo-Olivé et al. 2020). Since numerous begomoviruses share host and vector species, mixed infections are frequent and the potential to produce intra- and interspecific reassortants seems high. Consistently, well-documented recombinations and reassortments are known to impact begomovirus evolution (Pita et al. 2001; Saunders et al. 2002; Lefeuvre et al. 2009; De Bruyn et al. 2012; Lefeuvre and Moriones 2015; Fiallo-Olivé and Navas-Castillo 2023) even though mutation is reported to predominantly drive their diversification

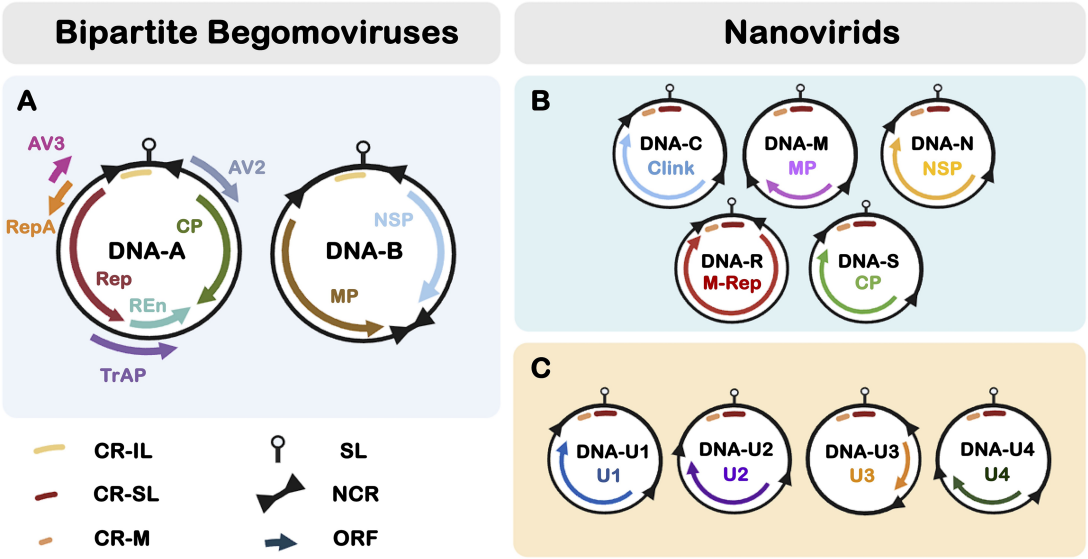

Figure 3. (A) Genome organization of a bipartite begomovirus. Both genomic components DNA-A and DNA-B are represented by a DNA molecule (black circle). The colored arrows represent the identified ORFs. The corresponding proteins are indicated with the same color as the arrows. Intergenic NCRs are represented overlapping the black circles. At the top of each DNA circle, a highly conserved stem-loop structure is represented. Rep: replication-associated protein; RepA: replication-associated protein A; CR-IR: common region of DNA-A and DNA-B corresponding to the highly conserved 200-nucleotide stretch within the large intergenic region of bipartite begomoviruses. (B and C) Genome organization of the *Nanoviridae* family. The five genomic segments (DNA-C, DNA-M, DNA-N, DNA-R, and DNA-S) shared between nanoviruses and babuviruses are shown in (B). The four genomic components specific to nanoviruses (DNA-U1, DNA-U2, and DNA-U4) and babuviruses (DNA-U3) are shown in (C). The name of each component is indicated within the corresponding circle. Colored arrows indicate the approximate size and position of ORFs with the corresponding name of the encoded protein accordingly indicated in the same color. At the top of each circle, two CRs (CR-M and CR-SL) including a stem-loop are represented inside the NCR. Rep: replication-associated protein; Clink: cell cycle link protein; U1, U2, U3, and U4: proteins of unknown functions.

(Lima et al. 2017). Moreover, due to the availability of multiple infectious clones, experimental reassortants are often produced to study the interactions of various species and isolates.

For brevity, we review later molecular constraints that could arise during the fundamental steps of replication, packaging, movement, and host-to-host transmission. We acknowledge that constraints could affect other aspects of the viral cycle. For example, the transcription factor TrAP borne by DNA-A was shown to regulate the expression of CP and MP ORFs borne by DNA-A and DNA-B, respectively (Sunter and Bisaro 1992; Hartitz, Sunter, and Bisaro 1999), and thus, because of the intersegment transcription regulation, could represent a significant associated constraint in reassortment.

Replication

DNA-A and DNA-B share one Conserved Region (CR-IR) in the Intergenic Non-Coding Region (NCR) that can form a stem-loop secondary structure with a highly conserved nonanucleotide loop sequence (5′-TAATATT/AC-3′) acting as the origin of replication (Argüello-Astorga et al. 1994; Laufs et al. 1995). It also contains a conserved sequence used to prime the synthesis of the complementary strand producing double-stranded DNA (dsDNA) replication intermediates (Saunders, Lucy, and Stanley 1992; Chandler et al. 2013; Bonnamy, Blanc, and Michalakis 2023). The origin of replication is cleaved between T and A (indicated above by a slash) in the nonanucleotide by the Rep protein to initiate replication (Laufs et al. 1995; Bonnamy, Blanc, and Michalakis 2023). The 3′ end of the cleaved strand serves as a primer for the host DNA polymerase (Wu et al. 2021) and the DNA synthesis proceeds, while the helicase activity of the Rep protein unwinds the dsDNA intermediate (Choudhury et al. 2006; Clérot and Bernardi 2006; Bonnamy, Blanc, and Michalakis 2023). When this polymerizing complex has copied the whole molecule and reaches again the origin of replication, the Rep protein ligates the 5′ and 3′ extremities of the newly formed ssDNA circle and the whole process, designated as Rolling-Circle Replication (RCR), can resume (Laufs et al. 1995; Bonnamy, Blanc, and Michalakis 2023).

Before cleaving within the nonanucleotide sequence, the Rep protein plays a key role in initiating replication by binding dsDNA at specific repetitive sequences called ‘iterons’ located in the CR-IR region (Fontes et al. 1994; Rizvi, Choudhury, and Tuteja 2015). These sequences act as replication specificity determinants (SPDs) and are present in iterative arrangements around the stem-loop structure (Argüello-Astorga et al. 1994). Over time, the length of iterons has been refined and now comprises short repeated sequences of 5–8 nucleotides (Argüello-Astorga and Ruiz-Medrano 2001). The sequence, number, and arrangement of iterons (relative position and spacing between them) can vary between genera and species (Argüello-Astorga et al. 1994; Fontenele et al. 2021).

The Rep protein is structured around several core functional domains. These domains are notably involved in DNA binding (Motif I), helicase (Motif II), and endonuclease activity (Motif III) (Ilyina and Koonin 1992; Orozco and Hanley-Bowdoin 1998). By analyzing more than 100 begomovirus iteron sequences and the corresponding associated Rep amino acid sequences, a hypervariable domain in the N-terminal region of the Rep protein was identified and called the Iteron Recognition Domain (IRD) (Argüello-Astorga and Ruiz-Medrano 2001). Interestingly, this variable Rep domain is conserved in species that share the same iterons, suggesting a role in their recognition (Argüello-Astorga and Ruiz-Medrano 2001). The IRD was defined as a stretch of less

than ten residues located in the twenty first amino acids of Rep that contributes to the folding of a conserved DNA-binding tertiary structure (Argüello-Astorga and Ruiz-Medrano 2001; Ramos et al. 2003; Londoño, Riego-Ruiz, and Argüello-Astorga 2010). Acting as a second recognition determinant, and also involved in the tertiary structure, two additional residues, approximately sixty AA downstream of the IRD, have been identified (Londoño, Riego-Ruiz, and Argüello-Astorga 2010; Avalos-Calleros et al. 2021). Distinct amino acids at these key positions recognize specific nucleotides, enabling prediction of potential iteron recognition based on the IRD sequence and as such of a potential match between the Rep encoded by a given DNA-A and a compatible CR-IR sequence in DNA-B (Argüello-Astorga and Ruiz-Medrano 2001; Gregorio-Jorge et al. 2010; Maliano et al. 2022).

The Iteron-Rep SPD matching predictions should be nuanced, as repeated iterons can be slightly different in the same molecule and also between DNA-A and DNA-B of the same genome (Maliano et al. 2022). Moreover, iterons can differ among closely related species and, conversely, distantly related species can share the same iteron sequences (Argüello-Astorga and Ruiz-Medrano 2001; Gregorio-Jorge et al. 2010). The Rep SPDs presumably allow for some variation in the sequences it recognizes, probably affecting its DNA binding (Fontes et al. 1994) and thus the efficiency of the associated DNA-B replication (Chakraborty et al. 2008).

The function of the begomovirus RepA involved in replication has not been clearly established (Rizvi, Choudhury, and Tuteja 2015; Sun et al. 2022a), whereas the REn protein, although not essential for replication, enhances it through interactions with Rep and host proteins (Sun et al. 2020). Consequently, from what is known so far, these proteins encoded by DNA-A- mostly interact with Rep, also encodedby DNA-A, and thus only interact with proteins encoded by the same segment. Any interactions these two proteins may have with proteins encoded by a reassorted DNA-B remain unknown.

Begomoviruses are reported to use a second replication process called recombination-dependent replication (RDR) (Jeske, Lütgemeier, and Preiß 2001; Preiss and Jeske 2003; Bonnamy, Blanc, and Michalakis 2023; this last reference contains schematic representations of the two replication mechanisms). This replication mechanism involves homologous recombination of a partially replicated ssDNA fragment, which inserts between the two strands of a circular dsDNA replication intermediate, anneals with the homologous region of the complementary strand, and primes the elongation process driven by a host polymerase (Bonnamy, Blanc, and Michalakis 2023). Depending on how far the elongation proceeds on this circular dsDNA intermediate template, linear dsDNA fragments of variable sizes are produced (Bonnamy, Blanc, and Michalakis 2023). These dsDNA linear fragments may then be used to initiate RCR if they encompass two origins of replication (Bonnamy, Blanc, and Michalakis 2023). The initial steps of the RDR are not driven by the Rep protein and do not rely on replicative determinants like iterons. The homologous recombination associated with the RDR process can occur not only anywhere between two copies of the same segment but also in the CRs between DNA-A and DNA-B. Consistently, recombination hotspots are detected in the CR-IR region (Lefeuvre et al. 2007, 2009; Martin et al. 2011b). Because DNA recognition target sequences or Rep SPD exchanges can enable new associations between DNA-A, DNA-B, and beta-satellites (Saunders et al. 2002; Chen et al. 2009; Silva et al. 2014), these hotspots near the origin of replication suggest that recombination and the RDR process can facilitate the production of viable reassortants. In the same way, recombination of DNA-A CR-IR in a newly reassorted DNA-B can result in an ameliorated match between DNA recognition targets, such as iterons, and Rep SPDs, facilitating its trans-replication (Hou and Gilbertson 1996; De Bruyn et al. 2012). Interestingly, monopartite begomoviruses, which are homologous to the DNA-A of bipartite species, have been suspected to capture DNA-B after recombination, thereby becoming bipartite (Saunders et al. 2002; Mansoor et al. 2003; Lefeuvre et al. 2007; Briddon et al. 2010; Ouattara et al. 2022). In the same line, several beta-satellites associated with monopartite begomoviruses have been reported to share identical DNA recognition targets with their helper viruses, which may have been acquired by the beta-satellites either through recombination with their helper or through gradual accumulation of mutations mimicking their helper's DNA recognition targets (Xu et al. 2019a).

Overall, a large proportion of the described begomovirus reassortants have close or identical iterons on their two genomic segments (Ramos et al. 2003; Bull et al. 2007; Singh, Chattopadhyay, and Chakraborty 2012; Silva et al. 2014). As a result, the compatibility between DNA recognition targets and Rep SPD amino acid sequences appears as a good predictor of the production of viable reassortants between different species and isolates (Gregorio-Jorge et al. 2010; Avalos-Calleros et al. 2021; Maliano et al. 2022). However, asymmetric patterns of reassortment where two species with compatible iterons can produce a viable A1–B2 reassortant but not the reciprocal A2–B1 are often observed (Hill et al. 1998; Garrido-Ramirez, Sudarshana, and Gilbertson 2000; Idris et al. 2008). Moreover, viable reassortants have been produced despite significant differences in iteron sequences or in iteron spatial arrangement (Garrido-Ramirez, Sudarshana, and Gilbertson 2000; Fontenele et al. 2021). For these cases, it should be noted that the structural details of iteron-Rep binding are not fully understood and that distinct combinations of both viral DNA and Rep protein may result in compatible binding and folding allowing trans-replication, likely with variable efficiency. Also intriguing is the fact that some seemingly iteron-independent replication has been reported for a delta-satellite where high-affinity binding between Rep and iterons was severely limited (Lin et al. 2003) implying the putative involvement of additional replication determinants allowing binding (Zhang et al. 2016) and additional use of RDR (Alberter, Ali Rezaian, and Jeske 2005).

Finally, there are also examples where viable or competitive reassortants cannot be produced despite similar or strictly identical iterons (Avalos-Calleros et al. 2021). These examples indicate that the iteron-Rep compatibility is necessary but not sufficient. Other factors involved in replication, such as putative additional regulatory sequences, or sequences in the Rep protein affecting its folding or binding properties, may also impact the stability of Rep-DNA or the recruitment of host polymerases. Unfortunately, unlike iterons, the other factors that could preclude replicative compatibility between two segments are not well characterized.

Packaging

Encapsidation plays a crucial role in the infection cycle of begomoviruses as it protects the viral genome, helps the importation of the ssDNA into the nucleus, and is a prerequisite, at least in most cases (Pooma et al. 1996), for long-distance movement (Jeffrey, Pooma, and Petty 1996) and plant-to-plant vector transmission (Czosnek et al. 2017). Therefore, successful packaging and assembly of stable particles are essential for the production of viable reassortants.

Geminiviruses are non-enveloped, and their genome is typically packaged within characteristic quasi-icosahedral

geminate particles, resulting from the fusion of two single quasi-icosahedrons together composed of 110 copies of the CP, each of symmetry $T = 1$ (Hipp et al. 2017; Hesketh et al. 2018). Cryo-Electron Microscopy structure analysis suggested that a 2.7-kb genomic DNA would fill the available internal space of geminate particles (Hesketh et al. 2018). Therefore, two such geminate particles are required to individually package the two segments of bipartite species (Hesketh et al. 2018). A minority of single and double icosahedrons, with the latter being unstable and probably rapidly disintegrating in single icosahedrons (Saunders et al. 2020), as well as single and triple quasi-icosahedron particles (Hooker and Salazar 1983; Frischmuth, Ringel, and Kocher 2001) have also been reported, all assembled from the same building-block coat protein (Saunders et al. 2020). Single icosahedrons can encapsidate smaller ssDNA circles that are generally defective-interfering or satellite molecules of approximately half the size of a genome component for alpha- and beta- to one quarter of the size for delta-satellites (Frischmuth, Ringel, and Kocher 2001; Casado et al. 2004; Jovel, Preiss, and Jeske 2007; Fiallo-Olivé et al. 2012; Saunders et al. 2020). Geminate particles can also encapsidate satellites (Hesketh et al. 2018; Saunders et al. 2020). What is encapsidated in the larger triple quasi-icosahedrons is unclear although it has been observed that begomoviruses often produce recombining defective ssDNAs of variable size that can exceed that of a full-length genomic segment (Jovel, Preiss, and Jeske 2007; Patil et al. 2007). In addition, experimental studies suggest that the begomovirus CP may be more permissive to the encapsidation of DNA molecules of different lengths than that of other geminiviruses, which require more precise genome-size molecules to ensure stable particle production (Saunders et al. 2020). This size-packaging flexibility in begomoviruses might facilitate the viable association with genomic components or satellites of variable length (Jovel, Preiss, and Jeske 2007; Patil et al. 2007; Fiallo-Olivé et al. 2012). Therefore, would the packaging step be a limiting factor for reassortment, it could only be due to a specificity between a given coat protein and the encapsidated sequences, for example, through the existence of specific packaging signals.

The N-terminal part of the coat protein of several geminiviruses has been found to contain a DNA-binding domain (Liu, Boulton, and Davies 1997; Qin, Ward, and Lazarowitz 1998), which allows the coat protein to bind to both ss- and dsDNA, in a sequence non-specific manner (Ingham, Pascal, and Lazarowitz 1995; Liu, Boulton, and Davies 1997; Palanichelvam et al. 1998; Hehnle, Wege, and Jeske 2004). First identified in the species *African cassava mosaic virus* (ACMV) (Hipp et al. 2017), a ring of basic residues, conserved in begomoviruses (Bennett and Agbandje-McKenna 2020), forms a pocket under the pentameric units interacting with the encapsidated DNA (Hipp et al. 2017). Cryo-EM structural analysis of the ageratum yellow vein virus particle determined that the electron density visible beneath each CP corresponded to a hexa- or hepta-nucleotide sequence (Hesketh et al. 2018). Interestingly, mutational analysis of AA residues within the DNA-binding site affected capsid assembly, highlighting the importance of CP–DNA interactions in this process (Hesketh et al. 2018). Encapsidation is believed to begin with no requirement for a specific DNA packaging signal, but uses genomic DNA as a scaffold enabling CP conformational changes necessary for the assembly of viral particles (Hesketh et al. 2018; Xu et al. 2019b). The interface between the two quasi-icosahedrons probably acts as an anchoring point for the viral DNA (Xu et al. 2019b). The DNA-binding sites within each CP subunit of a geminate particle together represent 110 potential inner contact points for the encapsidated DNA (Hesketh et al. 2018). Being single-stranded, segments may adopt variable secondary structures depending on their sequence and local physicochemical conditions. Distinct secondary structures may allow to attach variable proportions of these contact points, and thus, some segments might consolidate the viral particle more efficiently. Although this specific aspect has never been investigated, it may affect the particle stability and so eventually impact some of the reassortant properties.

In summary, the CP of begomoviruses can encapsidate a diverse range of ssDNA, both in length and sequence, and specific packaging determinants are lacking, suggesting that packaging is not a major constraint for the viability of reassortants.

Viral movement

Several proteins are involved in the movement of begomoviruses: (NSP)/BV1 and (MP)/BC1 proteins, encoded by ORFs borne by DNA-B, and (CP)/AV1 plus (PCP)/AV2 and AV3 (when present) (Ho, Kuchie, and Duffy 2014; Moshe et al. 2015; Gong et al. 2022), encoded by ORFs borne by the DNA-A component. Unfortunately, the mode of action of these proteins remains poorly understood.

The functional domains of NSP in begomoviruses have not been extensively studied (Sanderfoot, Ingham, and Lazarowitz 1996; Ward and Lazarowitz 1999; Zhou et al. 2007; Patil and Dasgupta 2022). Only two bipartite species have been investigated, *Squash leaf curl virus* (SqLCV) (Sanderfoot, Ingham, and Lazarowitz 1996) and *Indian cassava mosaic virus* (ICMV) (Patil and Dasgupta 2022). Two nuclear localization signals were identified in the NSP of SqLCV and ICMV, while an additional nuclear export signal was also found in ICMV NSP (Patil and Dasgupta 2022). Consistently, ICMV NSP was shown to localize both into the nucleus and at the cell periphery (Patil and Dasgupta 2022), whereas SqLCV NSP requires the co-expression of MP to exit the nucleus and move to the cell periphery (Sanderfoot, Ingham, and Lazarowitz 1996). The NSP protein is predicted to multimerize and is capable of binding both ssDNA and dsDNA in a size-dependent but sequence-independent manner (Rojas et al. 1998; Hehnle, Wege, and Jeske 2004). The role of NSP would be to shuttle the viral ssDNA strand to and from the nucleus. NSP and MP would form a complex either at the cell periphery (ICMV) or in/near the nucleus (SqLCV) and then translocate to the plasmodesmata for further cell-to-cell movement (Pascal et al. 1994; Sanderfoot and Lazarowitz 1995; Sanderfoot, Ingham, and Lazarowitz 1996; Patil and Dasgupta 2022). Depending on the species, MP is similarly capable of binding ssDNA and dsDNA in a size-dependent but sequence-independent manner (Pascal et al. 1994; Rojas et al. 1998; Hehnle, Wege, and Jeske 2004). It has been proposed that MP serves as a membrane anchor at the protoplasmic face of microsomes and plasma membranes, facilitating the movement of the NSP–DNA complex to reach plasmodesmata (Zhang, Ghosh, and Jeske 2002). In fact, two models have been proposed to explain the functions of MP in begomoviruses (Rojas et al. 1998; Jeske 2009). For phloem-limited species such as *Abutilon mosaic virus*, MP is thought to form a complex with NSP-bound viral DNA and localize along the plasma membrane before transferring NSP–DNA to adjacent cells (Hehnle, Wege, and Jeske 2004; Frischmuth et al. 2007; Jeske 2009). For mesophyll-invading begomoviruses like the bipartite species *Bean dwarf mosaic virus*, MP is thought to take over the viral DNA from NSP after being exported from the nucleus to the cytoplasm and deliver it to neighboring cells (Rojas et al. 1998).

Some bipartite begomoviruses also encode an AV2 protein, involved in gene silencing (Roshan et al. 2018; Basu et al. 2021),

coat protein production (Bull et al. 2007), viral accumulation (Padidam, Beachy, and Fauquet 1996; Bull et al. 2007; Roshan et al. 2018), and movement (Padidam, Beachy, and Fauquet 1996; Roshan, Kulshreshtha, and Hallan 2017; Roshan et al. 2018), but this latter function is still not fully understood (Rothenstein et al. 2007; Briddon et al. 2010; Roshan, Kulshreshtha, and Hallan 2017). Although monopartite begomovirus CP plays a crucial role in viral movement, in bipartite begomoviruses, it seems to be mostly involved in long-distance spread (Jeffrey, Pooma, and Petty 1996; Fondong 2013), probably through the production of virion progenies going through the sieve elements and indirectly due to its role in the protection and accumulation of ssDNA in the nucleus (Qin, Ward, and Lazarowitz 1998). The CP of some bipartite species is not necessary for systemic infection in specific hosts, but this remains the exception rather than the rule (Pooma et al. 1996; Sudarshana et al. 1998; Levy and Czosnek 2003).

Among the recently reported DNA-A new ORFs, AV3 might also function as a viral MP in monopartite begomoviruses. AV3 could localize at the plasmodesmata, traffic between cells, and was able to partially complement a potyvirus movement–deficient mutant (Gong et al. 2022). Interestingly, AV3 was first identified in a monopartite begomovirus, but is conserved in many bipartite species such as ACMV (Gong et al. 2021). Unfortunately, its role in viral trafficking remains largely unknown and appears to act on the host itself, independent of any interaction with other viral proteins. Therefore, its action in the movement of bipartite viruses is unlikely to impact the viability of a newly reassorted segment.

Together, the non-specific-sequence binding of CP, NSP, and MP to ssDNA and dsDNA and the 'autonomous' putative action of AV2 and AV3 suggest that the intra-host movement should not impose significant constraints on the viability of reassortants. However, functional complementation of DNA-B for different species infecting a common host was not always reciprocal, suggesting additional functional constraints that could limit reassortment (Frischmuth et al. 1993).

Host-to-host vector transmission

The coat protein has been found to be the sole determinant of vector transmission (Czosnek et al. 2017). Begomoviruses are typically transmitted in a circulative non-propagative manner, where virus particles are assumed to be the viral form cycling through the vector, without replicating (Wang and Blanc 2021). It is then assumed that the reassorted segment only needs to be efficiently packaged to be successfully transmitted.

Recent studies confirmed that the monopartite tomato yellow leaf curl virus (TYLCV) undergoes replication mainly in whitefly salivary glands (Pakkianathan et al. 2015; He et al. 2020). Would this be true for bipartite species, it might add interaction levels for the production of viable and transmissible reassortants. However, investigations with at least five other geminivirus species failed to reveal replication in their vector (Rosen et al. 2015; Wang and Blanc 2021), suggesting that only a small number of species may be able to replicate in whiteflies, only in specific organs and under certain conditions as this replication is negatively affected by stresses (Wang et al. 2016; He et al. 2020; Wang and Blanc 2021). In addition, while replication seems to contribute to TYLCV viral persistence in the vector (He et al. 2020), it is unclear how the virion progenies produced in insects contribute to transmission. Indeed, as for other species, most of the transmitted virus particles are assumed to be solely crossing the salivary gland cells through transcytosis.

In conclusion, while a DNA-A might not impose additional constraints on a reassorting DNA-B concerning the transmission step, it could nevertheless change some of its traits such as host range and vector specificity due to new CP properties (Höhnle et al. 2001; Idris et al. 2008; Fan et al. 2023) (Table 1).

Table 1. Interactions among proteins encoded by ORFs borne on different genomic segments.(1) Begomoviruses: Table overview of confirmed protein interactions involved in the corresponding viral functions borne either on DNA-A or DNA-B. (2) Nanovirids: Table overview of confirmed and suspected nanovirid protein interactions. Each column in order corresponds respectively to the concerned genomic segment, associated protein, (suspected)—detected interaction for *Nanovirus* or *Babuvirus* only, putative associated function.

(1)Begomoviruses			
Function	DNA-A	DNA-B	
Replication	Rep–RepA–REn	–	–
Encapsidation	CP	–	–
Viral movement	AV2–AV3–CP	MP–NSP	–
Transmission	CP	–	–
(2) Nanovirids			
Segment	Protein	Interactions	Function
C	Clink	–	Host manipulation
M	MP	NSP–U4–(CP)	Viral movement
N	NSP	CP–MP–M-Rep	Transmission
R	M-Rep	NSP–CP	Viral replication
S	CP	M-Rep	Encapsidation
U1	U1	–	Unknown
U2	U2	–	Unknown
U3	U3	–	Unknown
U4	U4	MP	Unknown

Natural population observations

The role of reassortment in the evolutionary dynamics of bipartite begomoviruses has been much less studied than recombination (Lefeuvre et al. 2009; Martin et al. 2011a, 2011b; Lefeuvre and Moriones 2015; Crespo-Bellido et al. 2021; Fiallo-Olivé and Navas-Castillo 2023). While numerous studies have investigated the genetic diversity of natural populations (De Bruyn et al. 2016; Crespo-Bellido et al. 2021), few studies undertook reassortment detection (Pita et al. 2001; Saunders et al. 2002; Briddon et al. 2010; De Bruyn et al. 2012; Chen et al. 2021; Xavier et al. 2021). Most of the related research focuses on experimental production of reassortants to evaluate the compatibility of isolates or species of interest in natural populations and anticipate potential agricultural threats (Crespo-Bellido et al. 2021; Fontenele et al. 2021; Maliano et al. 2022).

Among the few studies estimating reassortment frequency in natural populations, it has been reported that detecting reassortment events remained difficult and fluctuated depending on the sequence-analysis method (Xavier et al. 2021). This is mostly due to the limited number of sequences in datasets and the high nucleotide identity among exchanged components, making reassortments hard to detect. Hence, it seems apparent that reassortments occur preferentially between closely related begomoviruses (De Bruyn et al. 2012). This preference would reduce the disruption of intra-genomic interactions. Overall, these results stress the difficulty in identifying reassortment events amidst conserved sequences and most likely lead to an underestimation of their prevalence in natural populations.

Known studies identified 21–76 per cent of the described isolates, depending on the species, originating from at least one

reassortment event (Briddon et al. 2010; Xavier et al. 2021). Considering the above-mentioned potential underestimation, reassortment appears as a significant phenomenon in the ecological cycle of begomoviruses.

Conclusions

In conclusion, the constraints for reassortment in begomoviruses appear to be primarily related to trans-replication compatibility. Encapsidation shows a high degree of permissiveness, as observed associations between different genome sizes and stable capsid assembly do not rely on specific packaging signals. Viral movement relies on interactions between two MPs, NSP and MP, and their non-sequence-specific binding to viral DNA. Since both NSP and MP are present on the same genomic component (Table 1), their compatibility is not impacted by reassortment. The inconstantly present AV2 and AV3 genes seem involved in movement, but their function is still open to speculation. Lastly, vector transmission relies entirely on packaging, so reassortants capable of systemic infection and virion production are unlikely to face significant constraints for transmission, except concerning the inherent interactions between the capsid and vector proteins. Since all begomoviruses share the same whitefly vector, these interactions are likely complemented by most species. Even though only a relatively few studies report on reassortment frequency in natural populations, they reveal that the prevalence of reassortants is very high.

Nanoviridae—*Nanovirus* and *Babuvirus*

Introduction

The *Nanoviridae* family is composed of two genera. The *Babuvirus* genus comprises three species, namely, *Banana bunchy top virus* (BBTV) (Stainton et al. 2015), *Abaca bunchy top virus* (ABTV) (Sharman et al. 2008), and *Cardamom bushy dwarf virus* (CBDV) (Mandal et al. 2013), which infect monocotyledonous hosts from the *Musaceae* (BBTV and ABTV) and *Zingiberaceae* (CBDV) plant families. All known babuviruses are phloem-restricted and exclusively transmitted by aphid vectors, with *Pentalonia nigronervosa* being the most important species (Sharman et al. 2008; Mandal et al. 2013; Safari Murhububa et al. 2021). BBTV is notably responsible for the banana bunchy top disease, which is a highly devastating viral disease on banana crops in Asia and currently invading Africa (Dale 1987; Qazi 2016).

The *Nanovirus* genus comprises twelve species that infect dicotyledonous plants, mainly from the *Fabaceae* family (Lal et al. 2020). New species and isolates are reported frequently (Grigoras et al. 2014; Gallet et al. 2018; Vetten et al. 2019; Hassan-Sheikhi et al. 2020; Lotfipour et al. 2020; Sun et al. 2022b). Some of them increase the host range of the genus: an isolate of the faba bean necrotic yellows virus (FBNYV) can experimentally infect *Arabidopsis thaliana* from the *Brassicaceae* family (Vega-Arreguín, Gronenborn, and Ramírez 2007); several milk vetch dwarf virus (MDV) isolates infect tobacco plants from the *Solanaceae* family (Kamran et al. 2019) and garlic from the *Amaryllidaceae* family (Sun et al. 2022b); the new species *Parsley severe stunt–associated virus* (PSSaV) infects parsley from the *Apiaceae* family (Vetten et al. 2019; Hasanvand et al. 2021). This current host range expansion qualifies the genus *Nanovirus* as an emerging threat to the world agriculture (Lal et al. 2020). Just like babuviruses, all known nanoviruses are phloem-restricted and transmitted by several aphid species (Lal et al. 2020).

Nanovirids are the multipartite viruses with the highest number of genomic segments, six and eight for the *Babuvirus* and *Nanovirus* genera, respectively (Varsani et al. 2018) (Fig. 3). Each segment is a ssDNA circle of approximately 1 kb. Five segments are conserved in both genera: DNA-C encodes a protein interfering with the host cell cycle (Clink) (Aronson et al. 2000; Wanitchakorn et al. 2000; Lageix et al. 2007), DNA-M encodes the MP (Amin et al. 2011; Krenz et al. 2017), DNA-N encodes the helper component (HC) mandatory for aphid transmission (NSP) (Wanitchakorn et al. 2000; Grigoras et al. 2018), DNA-R encodes the replication initiator protein (M-Rep) (Timchenko et al. 2000; Horser, Harding, and Dale 2001), and DNA-S encodes the CP (Wanitchakorn, Harding, and Dale 1997; Trapani et al. 2023). An additional U3 segment is found in most babuviruses (Savory and Ramakrishnan 2014; Stainton et al. 2015), while additional U1, U2, and U4 segments are found in almost all known nanoviruses (Grigoras et al. 2014; Knierim et al. 2019; Hasanvand et al. 2021). The function of U1-U4 is presently unknown (Krenz et al. 2017). However, due to their consistent presence in natural isolates and their impact on several viral traits (Timchenko et al. 2006; Grigoras et al. 2014, 2018), they are considered as integral parts of the nanoviral genome. Nanoviruses are often found to be associated with alpha-satellites (Briddon et al. 2018). These are self-replicating molecules of approximately 1 kb encoding a protein homologous to M-Rep (Briddon et al. 2018; Kazlauskas, Varsani, and Krupovic 2018; Zhao et al. 2019). Nanovirus alpha-satellites are not believed to contribute any function to the other genome components as they do not trans-replicate segments of the helper virus (Timchenko et al. 1999); instead, they rely on it for encapsidation, movement, and transmission (Guyot et al. 2022; Mansourpour et al. 2022).

Each nanovirus species investigated has been consistently reported to differentially accumulate its genomic segments within host plants, yielding a pattern of segment frequency distribution designated as the 'genome formula' (Sicard et al. 2013, 2015; Yu et al. 2019; Bashir et al. 2022; Guyot et al. 2022; Mansourpour et al. 2022). This highly reproducible pattern is host-specific (Sicard et al. 2013, 2015) and has been interpreted as a means to rapidly tune gene expression through gene copy number variation in distinct host plant species (Zwart and Elena 2015; Gutiérrez and Zwart 2018; Gallet et al. 2022).

Infectious clones are available for several legume-infecting species and isolates of the genus *Nanovirus* as one clone per segment which can be inoculated as a mixture of clones via agroinfiltration (Grigoras et al. 2009, 2014). Experiments showed that segments R, S, and M are mandatory for systemic infection (Timchenko et al. 2006; Grigoras et al. 2018). The remaining segments are dispensable although their absence affects important viral traits: the absence of DNA-C reduces infection rate (Grigoras et al. 2018; Di Mattia et al. 2022), that of DNA-N abolishes aphid transmission (Grigoras et al. 2018; Di Mattia et al. 2020), and that of either U1 or U2 reduces viral accumulation, attenuates symptom severity, and may decrease infection rate (Timchenko et al. 2006; Grigoras et al. 2018). Intriguingly, the absence of U4 has no reported effect under laboratory conditions (Timchenko et al. 2006; Grigoras et al. 2018). Thus, its function remains a mystery, but as it is always present in field isolates (Grigoras et al. 2014), except in the recently discovered species PSSaV (Vetten et al. 2019; Hasanvand et al. 2021), it is assumed to play a role in particular in field conditions (Grigoras et al. 2018). Any of DNA-C, DNA-N, and DNA-U4 can be absent in systemically infected plants with no major phenotypic changes (Timchenko et al. 2006; Grigoras et al. 2018; Di Mattia et al. 2022) and few infected plants lacking these three segments could even be obtained (Timchenko et al. 2006), whereas infected plants with both U1 and U2 omitted at inoculation are extremely rare and show very mild symptoms

(Timchenko et al. 2006; Grigoras et al. 2018). Unfortunately, no infectious clones are available for the *Babuvirus* genus and thus analogous investigation of the dispensability of genome segments has not been conducted. Nonetheless, the absence of DNA-N in a field isolate has been reported (Fu et al. 2009), and symptoms were observed in two plants obtained under laboratory conditions, following aphid inoculation, where this segment remained undetected (Guyot et al. 2022). It should be noted that missing segments can be very common in nanovirid detection (Stainton et al. 2015; Knierim et al. 2019), including DNA-R, DNA-S, or DNA-M, which were shown to be mandatory in *Nanovirus*. It is very likely that these absences are due to the detection techniques as most of these samples are PCR amplified using degenerated primers, which can miss amplification and limit detection (Knierim et al. 2019). Field isolates when deeply sequenced after rolling-circle amplification reveal complete genomes with the exceptions of a few missing DNA-U4 (Knierim et al. 2019; Vetten et al. 2019; Hasanvand et al. 2021).

Experimental nanovirus reassortants were produced to study interspecific complementation. M-Rep proteins of the FBNSV, MDV, or subterranean clover stunt virus (SCSV) were able to trans-replicate DNA-S of any of these three species (Timchenko et al. 2000). A reassorted MDV DNA-S in a FBNYV genomic background was viable (Timchenko et al. 2006). FBNSV and FBNYV reassortants later enabled the identification of NSP as the 'HC' for transmission (Grigoras et al. 2018). The NSP from FBNSV was able to complement the transmission of a divergent (76 per cent amino acid identity between the two NSP) pea necrotic yellow dwarf virus (PNYDV) genomic background, but the reverse did not work. It is currently unknown whether this failure is due to a problem related to the reassorted segment helper capacity or to any other step in the viral life cycle (Grigoras et al. 2018). Therefore, under laboratory conditions, three essential nanovirus functions, replication, packaging, and vector transmission, can be complemented by distinct species, though with variable efficiency, which suggests relatively weak constraints on interspecific reassortment production.

Replication

All nanovirid genomic segments share two CRs in the NCR: Common Region (CR-SL) that can form a Stem-Loop with a highly conserved nonanucleotide sequence (5′ 'TAGTATT/AC' 3′) acting as the origin of replication (Hafner et al. 1997; Timchenko et al. 1999) and a major common region (CR-M) involved in the complementary strand synthesis by the plant machinery to produce dsDNA replication intermediates (Bonnamy, Blanc, and Michalakis 2023). No RDR (Jeske, Lütgemeier, and Preiß 2001) has been reported for nanovirids, but this may reflect a lack of relevant studies. Thus, nanovirids are presently assumed to replicate through the RCR process (Hafner et al. 1997; Timchenko et al. 1999). The Rep proteins of ssDNA viruses share a high structural similarity despite nucleotide and amino acid sequence divergence (Campos-Olivas et al. 2002; Vega-Rocha et al. 2007a; 2007b; Londoño, Riego-Ruiz, and Argüello-Astorga 2010; Kazlauskas et al. 2019; Venkataraman and Selvarajan 2019). Somewhat functionally supporting this structural observation, M-Rep appears to control an RCR mechanism similar to begomoviruses, with the involvement of DNA recognition target sequences, such as iterons and Rep SPDs (Herrera-Valencia et al. 2006; Stainton et al. 2017; Bonnamy, Blanc, and Michalakis 2023).

Three putative iteron motifs have been identified in the CR-SL of babuviruses, two located upstream and one downstream of the stem-loop (Herrera-Valencia et al. 2006; Stainton et al. 2017). Mutagenesis studies confirmed that changes in these iteron sequences can decrease or even abolish replication, depending on which iterons are modified (Herrera-Valencia et al. 2006). Alignment of all available babuvirus sequences (Stainton et al. 2017) has revealed some small differences in the iteron sequences not only among species but also among isolates and between segments within an isolate, suggesting that M-Rep may tolerate small sequence variations. Since these sequence differences are small, iterons are nevertheless quite similar across all three babuvirus species, also suggesting the possibility of heterologous trans-replication of most segments and a potentially relaxed constraint on both intra- and interspecific reassortments (Stainton et al. 2017). Unfortunately, in contrast to babuviruses, DNA recognition target sequences, such as iterons, have not been experimentally validated for nanoviruses. Nevertheless, repetitive and short palindromic sequences flanking the origin of replication are conserved between segments of the same species and may act as such in combination with uncharacterized DNA recognition motifs in the M-Rep (Timchenko et al. 2000; Grigoras et al. 2009; Londoño, Riego-Ruiz, and Argüello-Astorga 2010). Even though complementation was possible between FBNYV, MDV, and SCSV, observed quantitative differences in trans-replication were compatible with candidate iteron sequence divergences (Timchenko et al. 2000), suggesting a quantitative barrier to reassortment. Because proposed nanovirus iterons are not experimentally validated while several are short (3 or 4 nt), it is at this stage impossible to make solid predictions on the constraints of reassortment at this level.

Interactions between M-Rep/NSP and M-Rep/CP have been reported for the PNYDV in a leaf-infiltration system using fusions with reporter proteins (Krenz et al. 2017). Thus far, these putative interactions of M-Rep with other viral proteins are not understood and have not been detected in a natural viral infection context. Would they be validated, they could play a role in the regulation of replication and thereby impose a co-dependence between DNA-R, DNA-N, and DNA-S. Finally, it is unclear how the trans-replication of a reassorted segment might affect the genome formula and its consequences on reassortant fitness.

Packaging

With only two structural studies on nanovirids, the ssDNA packaging process is still largely unknown (Venkataraman et al. 2022; Trapani et al. 2023). The structural analysis of FBNSV (Trapani et al. 2023) was performed via cryoelectron microscopy with a 3D reconstruction model at atomic resolution applying the icosahedral symmetry. Consequently, the DNA densities within particles were averaged and not resolved, apart from the inner contact point with each of the sixty CP subunits which could be localized.

No packaging signal has been reported thus far in the CR shared among genomic segments. The genome packaging might thus rely mostly on non-specific interactions between the CP and viral DNA and impose no severe constraints to the reassorted segments. Comforting this hypothesis, a viable reassorted MDV DNA-S in a FBNYV genomic background was experimentally produced (Timchenko et al. 2006).

Viral movement

Again, not much is known or empirically confirmed about intra-host movement of nanoviruses. DNA-M has structural similarity with a geminivirus MP and thus likely encodes a protein of similar function (Burns, Harding, and Dale 1995; Sano et al. 1998). Fusion with fluorescent reporter proteins expressed in agro-infiltrated leaves of the non-host *Nicotiana benthamiana* demonstrated that the MP of a nanovirus (Krenz et al. 2017) as well as that of a

babuvirus (Zhuang et al. 2019) localized in cellular membranes, suggesting that it could travel to neighboring cells and to the vasculature via the symplastic route of the plasmodesmata (Zhuang et al. 2019). Very recently, CP amino acid substitutions preventing viral particle assembly, without affecting the CP/DNA interaction, proved to abolish systemic infection of FBNSV (Trapani et al. 2023). The authors concluded that full particle assembly and DNA encapsidation are likely required for long-distance movement. The two proteins MP and CP are involved, an interaction between the two may be required, and thus, the compatibility between DNA-M and DNA-S might impose constraints on the success of reassortments. A better understanding of the process underlying viral movement is thus necessary.

The protein encoded by DNA-N has been named NSP because Green Fluorescence Protein fusion experiments showed patterns of re-localization of the products of the DNA-N and DNA-M to the cell periphery (Wanitchakorn et al. 2000), similar to what was observed in begomoviruses (Rojas et al. 1998; Jeske 2009). As previously discussed, the NSP of begomoviruses has been experimentally shown to act in intracellular movement, in conjunction with the MP (Rojas et al. 1998; Jeske 2009). However, no implication of nanovirid NSP in movement has been experimentally confirmed, although an interaction between NSP and CP has been detected for BBTV relocating the CP to the cytoplasm from the nucleus (Ji et al. 2019). The fact that NSP omission at inoculation does not affect subsequent systemic symptom development and severity (Timchenko et al. 2006; Grigoras et al. 2018) is casting even more doubts on the contribution of NSP in within-host movement. Moreover, another function in vector transmission has been proven for this protein (see section Host-to-host vector transmission). In conclusion, the compatibility between MP and NSP or NSP and CP should not impose significant constraints for systemic movement of reassortants within the host plant.

Bimolecular fluorescence complementation analysis of PNYDV-infiltrated *N. benthamiana* leaves detected MP–MP and MP–U4 interactions, inducing in both cases localized fluorescent spots at the nuclear membrane, associated with the endoplasmic reticulum and at the cell periphery close to plasmodesmata (Krenz et al. 2017). MP and U4 are predicted to have a transmembrane domain and their co-localization near plasmodesmata suggests that they may form a complex at these sites, although definitive proof is lacking (Krenz et al. 2017). Since U4 is dispensable under laboratory conditions (Grigoras et al. 2018), and in the case of PSSaV also in natural infections (Vetten et al. 2019; Hasanvand et al. 2021), the requirement of a compatible interaction between DNA-U4 and DNA-M encoded proteins remains to be confirmed. Whether a putative constraint exists at this level upon reassortment awaits additional functional information.

Host-to-host vector transmission

Nanovirids are transmitted in a circulative non-propagative manner (Hafner, Harding, and Dale 1995; Sicard et al. 2015; Wang and Blanc 2021). One peculiarity that differs from begomoviruses is that the purified nanovirus particles cannot be acquired and transmitted by the insect vectors (Franz et al. 1999). A HC produced in infected host plants is necessary to complement the aphid transmission of purified virus particles. This HC has recently been identified as the product of DNA-N (Grigoras et al. 2018). Aphid vectors fed on BBTV-infected plants missing DNA-N were unable to transmit the virus, suggesting a function as HC also for babuviruses (Guyot et al. 2022). The interaction between NSP and CP identified for the BBTV (Ji et al. 2019) remains undetected for any species of the *Nanovirus* genus (Krenz et al. 2017).

All described HCs, in many unrelated viral taxa such as, for example, the genera *Caulimovirus, Potyvirus*, or *Tenuivirus* recently reviewed (Di Mattia et al. 2023), connect the virus particles to the receptor in the insect vector, via two distinct functional domains, one interacting with the coat protein and the other with the receptor molecule. Although its putative receptor in aphids is unknown, NSP has been shown to be mandatory for the internalization of the virus particles of FBNSV in midgut cells, where viral DNA, coat protein, and NSP colocalize and accumulate in cytoplasmic membrane–bound inclusions (Di Mattia et al. 2020, 2022). The nanovirus NSP appears to have a unique property that can impact reassortment (Di Mattia et al. 2022). Due to their mode of action, first recognizing a receptor in the vector and then bridging the virus particles, all characterized HCs must be acquired prior to (or together with) the virus particles for successful transmission (Di Mattia et al. 2023). Intriguingly, both acquisition orders proved to be efficient for the transmission of FBNSV, enabling complementation of the transmission of genomic components acquired either a few days before or after NSP (Di Mattia et al. 2022). This phenomenon opens a 'window' of several days where the acquisition of a DNA-N renders the aphid competent for acquiring additional segment sets on other host plants, even if these are not transmissible on their own (Di Mattia et al. 2022, 2023).

Natural population observations

Investigations in babuvirus natural populations have detected multiple isolates with shared reassortment events (Stainton et al. 2012, 2015; Savory and Ramakrishnan 2014). Studies of BBTV and CBDV isolates established that 40 per cent had undergone at least one reassortment (Savory and Ramakrishnan 2014; Stainton et al. 2015). Despite frequent reassortment events for CBDV and BBTV, there is a noticeable absence of interspecific reassortants (Stainton et al. 2012, 2015; Savory and Ramakrishnan 2014). Interestingly, most of the detected intra-segment recombination events involve different species, indicating that the rarity of interspecific reassortments cannot be attributed to limited encounter of parental genotypes.

Only two comprehensive sequencing studies were conducted on natural populations of nanoviruses. One compared multiple isolates of eight species and identified twelve reassortment events, eleven involving only one segment and one involving two segments (Grigoras et al. 2014). Only two reassortment events involved different species, indicating that, as already noted for babuviruses, successful reassortment events are more likely to occur within a species. This study also detected twenty-three recombination events, with eighteen occurring between different species, again suggesting that encounters between parental genotypes of distinct species cannot explain the relative paucity of interspecific reassortants. The second study compared sixteen complete isolates, mostly of FBNYV (Kraberger et al. 2018), and detected ten reassortment events, mainly intraspecific, with only one involving two segments. For both genera, the survey size was limited. In all cases, however, multiple species co-occurred in the same geographical area and shared host species (Stainton et al. 2012, 2015; Grigoras et al. 2014; Savory and Ramakrishnan 2014; Kraberger et al. 2018). In this regard, the relative scarceness of interspecific reassortments is remarkable and should be investigated further.

In babuviruses, although all segments were involved in at least one reassortment event, some segments were more often involved than others (Table 2). Over several studies (Stainton et al. 2012, 2015; Savory and Ramakrishnan 2014) DNA-M, DNA-N, and DNA-U3 proved to be more prone to reassort, than DNA-R

Table 2. Summary of nanovirid natural population surveys and reassortment detections.

Genus	Complete genomes	Events	C	M	N	R	S	U1	U2	U3	U4	Ref
Babuvirus	17 BBTV	8	0 (0%)	3 (38%)	2 (25%)	2 (25%)	1 (13%)	–	–	1 (13%)	–	Stainton et al. 2012
Babuvirus	121 BBTV—2 ABTV	40	5 (13%)	8 (20%)	7 (18%)	2 (5%)	7 (18%)	–	–	11 (28%)	–	Stainton et al. 2015
Babuvirus	163 CBDV	23	2 (9%)	7 (30%)	7 (30%)	2 (9%)	2 (9%)	–	–	2 (9%)	–	Savory and Ramakrishnan 2014
Nanovirus	3 BMLRV—6 FBNSV—13 FBNYV—1 FBYLV—1 MDV—1 PYSV—2 PNYDV—2 SCSV	12	0 (0%)	4 (33%)	2 (17%)	2 (17%)	0 (0%)	0 (0%)	4 (33%)	–	1 (8%)	Grigoras et al. 2014
Nanovirus	16 FBNYV	10	0 (0%)	3 (30%)	2 (20%)	1 (10%)	1 (10%)	3 (30%)	1 (10%)	–	0 (0%)	Kraberger et al. 2018

Columns from left to right correspond to the genus, the number of complete genomes per surveyed species, the number of individual reassortment events detected in the study, how many were associated with the indicated genomic segment, and finally the corresponding publication. Full names of viruses not mentioned elsewhere in the text are BMLRV: black medic leaf roll virus, FBYLV:faba bean yellow leaf virus, and PYSV: pea yellow stunt virus

and DNA-S. DNA-C was the segment which reassorted less in all cases (Table 2). In nanoviruses, all segments except DNA-C were detected in reassortants (Table 2). Specifically, DNA-M reassorted most, followed by DNA-N, DNA-R, and DNA-U2, while DNA-U1 and DNA-U4 were sometimes not involved in reassortments at all. Since this is based on only two studies with a small number of genomes (Grigoras et al. 2014; Kraberger et al. 2018), the conclusions are limited and do not account for the recent expansion of available nanovirus sequences. However, in both genera, DNA-M and DNA-C are highly and poorly reassorting, respectively.

Genomic segment sequence conservation across species and genera might affect the reassortment frequencies as most conserved segments probably reassort more easily. Nanovirus studies showed three groups of segment conservation: DNA-R, DNA-S, and DNA-N are highly conserved at the species (~90 per cent pairwise identity) and genus levels, followed by DNA-C, DNA-U1, DNA-M, DNA-U2, and DNA-U4. Thus, at the intraspecific level, sequence divergence does not seem to explain reassortment prevalence as DNA-C is more conserved than DNA-M but has not been involved in any reassortment even described so far contrary to DNA-M, which is the most involved segment. DNA-R stands as the most conserved segment in the genus, with around 76 per cent pairwise identity between the two most divergent species. This conservation is less contrasted for the other genomic components, which share approximately 60 per cent pairwise identity between the most divergent species (Grigoras et al. 2014; Kraberger et al. 2018). From a large BBTV study, on average, DNA-R (>88 per cent) and DNA-C (>85 per cent) appear to be the most conserved inside the species followed by DNA-N (>83 per cent), DNA-S (>82 per cent), DNA-M (>82 per cent), and DNA-U3 (>74 per cent) (Stainton et al. 2015). Again, the degree of sequence conservation does not match sequence implication in reassortments (Table 2).

Conclusion

Although our understanding of the molecular processes in nanovirids is currently limited, reassortment constraints likely depend heavily on the trans-replication of the reassorted segment through compatibility between M-Rep SPDs and DNA recognition target sequences, for instance, iterons. However, despite the limited diversity of babuviruses, the replication complementation between divergent nanovirus species and the small range of identified babuvirus iteron sequences may allow for more relaxed constraints than in begomoviruses. We presently do not know how replication of distinct segments with small differences in DNA recognition target sequences might affect intra-genomic interactions. Packaging constraints in nanovirids remain largely unknown, as no studies have been conducted on the interaction between the coat protein and DNA or packaging itself. However, frequent association with alpha-satellites and experimental observations showing the viability of an interspecific reassortment of DNA-S suggest a permissive process. Concerning viral movement, interactions involving DNA-M, DNA-S, DNA-N, and DNA-U4 may be necessary and may significantly limit the viability of reassortants (Table 1). However, the frequent loss of DNA-N and DNA-U4 under experimental conditions, along with the absence of detected interactions between DNA-M and DNA-S, suggests that these constraints may not be as important as initially thought. Regarding transmission, nanovirids heavily rely on both DNA-S for stable virion production and DNA-N for the HC NSP required to penetrate the aphid midgut barrier. Reassortment of DNA-N might therefore impose a severe constraint on reassortant transmission. However, once again, the experimental functional complementation between divergent species and the shared aphid vectors indicates degrees of permissiveness. Furthermore, the window for complementation of vector transmission opened by the peculiar mode of action of NSP likely enhances virus capacity for reassortment. Although natural observations confirm a relatively high frequency of reassortment in nanovirids, a striking discrepancy is present between assumptions based upon relaxed constraints on interspecific reassortments and their rarity in the field. Similarly, the scarcity of reassortments involving more than one segment is intriguing.

Other multipartite ssDNA viruses

The other ssDNA viruses that are known or strongly suspected to have a multipartite genome organization can be distinguished into two categories. The first one regroups viral genomes probably

replicated by a protein of the Rep family according to the RCR mechanism, similar to the begomo- and nanoviruses detailed earlier. These include the plant-infecting Coconut foliar decay virus, sole member of the *Cofodevirus* genus within the *Metaxyviridae* family (Gronenborn et al. 2018), and the first ssDNA multipartite virus described in fungi, Fusarium graminearum gemytripvirus 1, sole member of the *Gemytripvirus* genus within the *Genomoviridae* family (Li et al. 2020). Both these viruses have three circular ssDNA genomic segments with a conserved stem-loop region analogous to the origin of replication of gemini- and nanoviruses, but very little information is available on the molecular biology of their infection cycle. Thus, apart from the necessary match between the Rep protein SPDs and the conserved DNA recognition target sequences containing the region encompassing the origin of replication (Gronenborn et al. 2018; Li et al. 2020), how the other putative limitations to reassortment detailed earlier apply to these viruses is impossible to predict. Additional sequences become available every day, and it is most likely that countless similar multipartite circular Rep-encoding ssDNA (CRESS) viral species will be characterized in the future (Male et al. 2016; Kraberger et al. 2019), perhaps infecting a wider range of hosts where the role or consequences of reassortment will be most interesting to address.

The second category of multipartite ssDNA virus is represented by two animal infecting species: *Bombyx mori bidensovirus* (BnBDV), only representative species of the *Bidensovirus* genus in the *Bidnaviridae* family (ZhaoYang et al. 2013), and *Acheta domesticus segmented densovirus* representative of the *Brevihamaparvovirus* genus in the *Parvoviridae* family (Pénzes et al. 2023). The genomes of these two viruses consist of two linear ssDNA genomic segments (Wang et al. 2006; Pénzes et al. 2023). Molecular processes of the infection cycle of bidensoviruses and brevihamaparvoviruses, encompassing replication (Tijssen and Bergoin 1995; Zhaoyang et al. 2016), packaging (Pan et al. 2014; Lü et al. 2017), movement, and transmission, are poorly documented and could be very different from those of the plant-infecting CRESS viruses previously described. Unfortunately, despite evidence of co-occurrence of distinct BnBDV isolates (Gani et al. 2021), the existence and properties of putative reassortants have never been investigated, neither under laboratory conditions nor in natural populations.

Discussion

Among the reviewed molecular processes, replication compatibility appears to be the primary factor determining the production of viable reassortants. This is because trans-replication of heterologous segments mostly depends on specific recognition of DNA target sequences by the Rep protein for CRESS viruses, i.e. all ssDNA multipartite viruses up to date except for bidensoviruses and brevihamaparvoviruses. Updating the precise correspondence between DNA recognition target sequences and the Rep SPDs in known reassortants would greatly enhance our capacity to forecast potential associations. This is particularly relevant for begomoviruses, as no SPDs have yet been confirmed on the M-Rep protein of nanovirids, although they probably exist (Londoño, Riego-Ruiz, and Argüello-Astorga 2010). Additionally, the characterization of nanovirus iterons remains incomplete, restricting our understanding of their trans-replication capacity. While babuvirus iterons have been more extensively characterized, with only three known species conclusions on their diversity are limited.

In contrast to replication, packaging and intra-host movement do not seem to impose major constraints on reassortment since they involve mostly non-specific binding to viral DNA. However, despite the permissiveness of their CP to the encapsidation of variable size DNA components, begomoviruses incur limitations to their genome size imposed by NSP, MP, and plasmodesmata (Gilbertson et al. 2003) as both NSP trafficking and MP trafficking are optimized around the size of genome molecules (Rojas et al. 1998; Gilbertson et al. 2003). Limitations related to intracellular movement could arise if interactions among viral proteins carried by separate segments were involved. Current knowledge suggests that for begomoviruses, the focus is mainly on interactions between NSP and MP, both of which are present on DNA-B, and potentially CP, carried by DNA-A (Table 1). Therefore, except when the CP is also involved, this step of the viral life cycle should not represent a significant impediment to reassortment. However, the functional complementation of DNA-A movement by a DNA-B from a different species demonstrated certain limitations as reciprocal complementation for multiple species was not possible (Frischmuth et al. 1993). In the case of nanovirids, compatibility between MP, U4, and CP may be required, but the processes involved in movement necessitate more investigations.

Host-to-host transmission is another life cycle step that should not represent a major hurdle to reassortment. For begomoviruses, transmission predominantly relies on viral particles, making it largely reliant on the assembly and packaging of virions. Therefore, if a reassortant begomovirus can successfully complete the packaging step, transmission should not pose additional problems. In contrast, nanovirids require a HC, the NSP protein, but interspecific complementation of the NSP function is possible under laboratory conditions (Grigoras et al. 2018). A relevant and so far unique aspect of the helper function of nanovirids is that NSP can complement the transmission of viral particles that have been acquired by the vector at least a few days earlier (Di Mattia et al. 2022), greatly enhancing the temporal range of complementation and thus successful reassortment.

Confronting predictions based on molecular constraints with field data is more complicated for bipartite begomoviruses than for nanoviruses, since nanoviruses have six to eight genomic segments and mostly a one-function-one segment genome organization. This makes it possible to identify a major and a minor parent based on their relative segment contribution and, more to the point, to identify segments and functions more or less involved in reassortments and constraints. This is not the case for bipartite begomoviruses, not only because with only two segments, it is impossible to tell which segment is more or less involved in reassortment, but also because in begomoviruses, many functions are encoded on the same segment (Table 1), which makes it a priori difficult to ascribe constraints on specific functions. Nevertheless, numerous reassortment events in the few available surveys reveal high reassortment prevalence, which may suggest a relevant role in genome reconstitution, that is, the possibility that DNA-A and DNA-B may be often transmitted separately from cell to cell or even host to host.

The nanovirus experimental functional complementations, the limited diversity of known babuvirus iterons, and the existing ecological opportunities would predict relatively high prevalence of interspecific reassortments in nanovirids. However, this is not consistent with field data where interspecific reassortants are puzzlingly rare relative to the abundance of interspecific recombinants. Because recombination requires co-infections at the individual cell level, while reassortment in nanovirids may occur even in the absence of co-infection of the same individual host, the relative prevalence of interspecific recombinants strongly suggests that the rarity of reassortants is not due to a lack of opportunity of encounter between viral genotypes but rather to significant constraints on interspecific reassortant viability or competitivity.

This striking imbalance between interspecific reassortants vs recombinants is intriguing and begs for an explanation. The availability of infectious clones for several nanovirus species, and the possibility to produce new ones for additional isolates of the same species, opens the opportunity to further investigate and compare the properties of inter- and intraspecific reassortment in the laboratory. Beyond viability, a prominent question would then be the comparison of their fitness relative to that of parental genotypes.

As incomplete sets of segments can be independently transmitted between hosts, missing segments, with nanoviruses having at least three non-essential segments under laboratory conditions, should allow for the complementation of multiple segments at once. Yet, another intriguing aspect of nanovirid reassortments is the almost exclusive representation of single-segment reassortment events in most studies, which seems to indicate significant genome disruption when more than one segment is involved. This would limit the scope of complementation of incomplete genomes and could be experimentally investigated by the evaluation of multiple-segment reassortant fitness.

As mentioned earlier in this review, there is a discrepancy between the frequently observed loss of non-essential segments under laboratory conditions and the prevalence of complete genomes in deeply sequenced field samples (Knierim et al. 2019). One possible explanation could be that some important conditions in maintaining all segments in the field are relaxed in the laboratory. For example, nanoviruses may switch host very often *in natura*, with some segments being mandatory depending on the host. Another possible explanation could be that field ecological dynamics involving hosts, vectors, and viruses lead to frequent reconstitutions, rendering the existence of incomplete infections extremely transient and masking a recurrent but reversible loss of segments. In this regard, segment reassortment frequency might shed some light on the complementation dynamics in natural populations and should in principle reveal more frequent reassortments for dispensable segments. From the limited number of relevant studies, all segments except DNA-C have been involved in reassortment events (Table 2). In particular, there is no bias toward non-essential segment reassortments. This may also suggest that delayed complementation, enhanced by the action of nanovirus NSP, could occur and potentially rescue incomplete 'latent' infections missing essential segments, if these viral particles can 'wait' in their host and vector for a sufficient amount of time (Michalakis and Blanc 2020). This possibility of a latent phase for incomplete sets of segments lacking an essential function warrants further investigation as it could further enhance the potential to reassort and further reduce the cost of maintaining genomic integrity.

An additional point where predictions based on laboratory experiments with nanovirids and field observations do not match concerns how molecular constraints might affect the reassortment frequency of associated segments. Replication stands out as a key step limiting reassortment, and we would thus expect DNA-R to be the least reassorting segment. Surprisingly, DNA-R does not stand out as a poorly reassorting segment, while DNA-M, another 'essential' segment, for which an interaction with CP is suspected, is the most frequently reassorting in both genera (Table 2). The third essential segment, DNA-S, which is expected to require compatible interactions with NSP and MP, is roughly reassorting as frequently as DNA-R (Table 2). The case of the non-essential DNA-C is even more intriguing. As it is supposed to modulate the host cell cycle, it should not impose serious limitations for reassortments. Yet, DNA-C has so far never been found reassorting in nanoviruses and to a limited extent in babuviruses (Table 2). Highly conserved segments could reassort more easily than expected from their implication in molecular constraints. This could be the case for the very conserved DNA-R, which is found relatively frequently in reassortants (Grigoras et al. 2014; Stainton et al. 2015; Kraberger et al. 2018) despite its implication in trans-replication, the largest molecular constraint. However, sequence conservation cannot explain why DNA-M and DNA-C differ so much in their reassortment prevalence as DNA-C is more conserved but reassorts less.

In conclusion, reassortment appears to be an important phenomenon in ssDNA multipartite viruses. However, experimental predictions, especially for nanovirids, do not align with natural population observations, indicating that there are likely major gaps in our understanding of the molecular processes, the ecological dynamics, and the phenotypic effects. Conducting a large and systematic study on the fitness of reassortants of ssDNA multipartite viruses and their parental genotypes would help bridge this gap.

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Data availability

There are no new data associated with this article.

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