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BRIEF REPORT

Phylogenetic relationships and the occurrence of interspecific recombination between beet chlorosis virus (BChV) and Beet mild yellowing virus (BMYV)

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Abstract Samples containing two viruses belonging to the genus *Polerovirus*, beet chlorosis virus (BChV) and beet mild yellowing virus (BMYV), were collected from French and Polish sugar beet fields. The molecular properties of 24 isolates of BChV and BMYV were investigated, and their genetic diversity was examined in the coat protein (CP)- and P0-encoding genes. For the first time, we have demonstrated that beet polerovirus populations include recombinants between BChV and BMYV containing breakpoints within the CP gene. Moreover, a partial correlation between geographic origin and phylogenetic clustering was observed for BMYV isolates.

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Three aphid-transmitted poleroviruses (family *Luteoviridae)* have been described as causal agents of mild yellowing of sugar beet: beet mild yellowing virus (BMYV), beet chlorosis virus (BChV) and American isolates of beet western yellows virus (BWYV-USA) [1–3]. These so-called 'beet poleroviruses' are widespread throughout the world [4, 5], causing serious economic damage in sugar production [6]. They are closely related to the non-beet-infecting turnip yellows virus (TuYV), which belongs to a separate species in the genus *Polerovirus* [7]. These viruses are restricted to the phloem tissue and have various host ranges. Their virions are icosahedral particles containing a single-stranded positive-sense RNA organized in six open reading frames (ORF). The variety of genome expression strategies used by members of the family *Luteoviridae* contributes to the adaptability of the virus to new environments, host plants and vectors [8]. ORF0 encodes a suppressor of post-transcriptional gene silencing (PTGS) [9, 10]. P1 and P2 proteins are required for viral replication [8]. ORF4 encodes a putative movement protein [11]. The major coat protein (CP) and the readthrough (RT) protein (P3-P5 fusion protein) are both involved in virus acquisition, circulation and inoculation by aphid vectors [12]. Analysis of nucleotide and amino acid sequences of distinct beet polerovirus isolates originating from various countries revealed the presence of highly conserved regions in CP (reaching 90 % similarity) among BMYV isolates [13]. Conversely, the sequence of ORF0 is highly variable. The conserved features have been used for the development of generic (CP gene) and specific (P0 gene) RT-PCR tools to detect the three 'beet poleroviruses' [1, 3].

Populations of most plant viruses are genetically heterogeneous, and recombination is considered to play a major role in virus variability and thus in virus evolution [13–15].

Table 1 Geographical origin, and year of sampling of beet polerovirus isolates and accession numbers of the CP and P0 gene sequences

Virus isolate	Country, region/department/ voivodeship	Year	CP or P5* sequence accession number	P0 sequence accession number
BChV-2a	Great Britain, Suffolk	1997	AF167475	AF168610
BChV-18 K	Poland, Warsaw	2005	EU022509*	EF659974
BChV-G43	France, Pas-de-Calais	2005	EU022495	EU022472
BChV-M26	France, Somme	2005	EU022510*	EU022464
BChV-M27	France, Somme	2005	EU022490	EU022465
BChV-N1	France, Pas-de-Calais	2005	EU022491	EU022466
BChV-N5	France, Pas-de-Calais	2005	EU022492	EU022467
BChV-N7	France, Pas-de-Calais	2005	EU022493	EU022468
BChV-N13	France, Pas-de-Calais	2005	EU022511*	EU022469
BChV-No33	France, Haute Normandie	2005	EU022494	EU022470
BChV-O36	France, Oise	2005	JX 465678	EU022471
BMYV-2ITB	France, Eure et Loir	1980	X83110	X83110
BMYV-19K	Poland, Warsaw	2005	EU148508*	EU022479
BMYV-26	Poland, Bydgoszcz	2005	EU022504	EU022485
BMYV-D13	Poland, Kutno	2006	EU022503	EU022484
BMYV-G44	France, Pas-de-Calais	2005	EU022502	EU022473
BMYV-Md	Poland, Warsaw	2006	EU022507	EU022488
BMYV-M5	Poland, Warsaw	2006	EU022505	EU022486
BMYV-M8	Poland, Warsaw	2006	EU022506	EU022487
BMYV-N9	France, Pas-de-Calais	2005	EU022498	EU022478
BMYV-N18	France, Pas-de-Calais	2005	EU022499	EU022475
BMYV-N20	France, Pas-de-Calais	2005	EU022496	EU022480
BMYV-N27	France, Pas-de-Calais	2005	EU148509*	EU022481
BMYV-N32	France, Pas-de-Calais	2005	EU148510*	EU022476
BMYV-O37	France, Oise	2005	EU022497	EU022482
BMYV-Th	France, Pas-de-Calais	2006	EU022501	EU022474

The isolates used as references are in bold

Recombination events between ancestors of members of the genera *Polerovirus* and *Luteovirus* have been hypothesized to explain the emergence of new viruses in the family *Luteoviridae* [16, 17]. In this study, we assessed the genetic diversity of beet polerovirus isolates sampled from several French and Polish sugar-beet-growing areas. To estimate their genetic variation, sequence analyses were focussed on two different parts of the genome: the highly conserved structural CP gene and the more variable non-structural P0 gene.

During three years of epidemiological study, 336 leaf samples in Poland and 64 in France were collected from apparently symptomatic sugar beet plants, except for the BMYV-19K and BMYV-26 isolates, which were collected from red beet and fodder beet, respectively. The origins of the beet polerovirus isolates that were sequenced are given in Table 1. Isolates BMYV-2ITB and BChV-2a, which were maintained on sugar beet plants (*Beta vulgaris* cv. Trestel) [4, 18], were used as reference isolates. All other isolates that were used for analysis were obtained directly from the field. The collected samples were frozen (−20 °C) in aliquots prior to RNA extraction, RT-PCR and immunoassay experiments.

Beet poleroviruses were detected in leaf samples, either by triple antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody MAFF 24, raised against BMYV-1 isolate from the UK [5], as described previously [19], or by double antibody sandwich (DAS)-ELISA using polyclonal antibodies obtained from Loewe Biochemica GmbH (Sauerlach, Germany). The samples that tested positive in ELISA were subjected to total RNA extraction using an RNeasy Plant Mini-Kit™ (QIAGEN). The single-tube RT-PCR multiplex protocol (Ready-To-Go RT-PCR Beads™, Amersham-GE Healthcare) and the specific primers Mpx BC+/BC− or MpxBM+/BM, described by Hauser *et al*. [3], were used to identify poleroviruses.

To amplify the P0, CP and RT genes for sequencing, two-step RT-PCR was used. Total RNAs of selected samples were reverse transcribed using MMLV reverse

transcriptase (Promega) and specific reverse primers. cDNA were then amplified by PCR (HotGoldstar, Eurogentec). For detection of recombination events, RT protein was amplified for BChV using the following primer sets in the 5′-3′ orientation: RT1+/RT1− (AAGGCAATGGTTC TTCATCG/GTTCCATGTCCGGGTGTC), RT2+/RT2− (CTCAAGGAAGGTTGGAACG/GGAAGGGGCAAGTC TCTC), RT3+/RT3− (GGGCATCGAGAAGAGAGAC/T TCATCAGGACCAGAAAGGG). For BMYV, the primers RT1+/RT1− (AAGGCAATGGTTCTTCATCG/ACGTAG CAAGTCAAATCTCC), RT2+/RT2− (TGGAGATTTG ACTTGTCACG/GCCATGCCTCAACCAAG), RT3+/ RT3− (GCTGCGTCATCAAAGAGTG/AAGTGCCGTA GGGAGTTATC) were used. The beet-polerovirus-specific primer pair CP+/CP−, as well as primers specific for the BChV or BMYV P0 protein were designed previously by Hauser *et al*. [7]. RT-PCR products were then purified (QIAquick PCR Purification Kit^{TM}, QIAGEN) and were sequenced bidirectionally two or three times to ensure reliable sequence data. These sequences have been submitted to the GenBank database, and their accession numbers are shown in Table 1.

Nucleotide and amino acid sequences were analyzed using Vector NTI, version 7.1, and the MEGA6 package [20]. Sequences were assessed using Clustal X^{TM} [21]. Phylogenetic trees were constructed using maximum-likelihood, neighbor-joining and minimum-evolution algorithms, and the best-fitting model was estimated in MEGA6. We assessed confidence of branching patterns in the phylogenetic tree by the bootstrap method, with 1000 pseudo-random replicates.

Genetic distances were determined using the Kimura 2-parameter model with gamma distribution (K2 + G). The occurrence of suspected recombinants was confirmed with the program SISCAN 2.0 [22]. This algorithm calculates Z-values for pairwise identity scores of aligned sequences of the putative recombinant and its two putative parents within a sliding window (100 nt) that moves by steps of 50 nt. The GARD (genetic algorithm recombination detection) and SBP (single breakpoint recombination) methodologies were also used to determine whether recombination events were present in the sequences studied [23, 24]. Selection pressure analysis was conducted in Datamonkey for the CP and P0 genes [24]. The following methodologies were used: SLAC (single-likelihood ancestor counting), FEL (fixed effects likelihood) REL (random effects likelihood) and MEME (mixed effects model of evolution). The proportion of non-synonymous substitutions (dN) versus synonymous substitutions (dS) was estimated in MEGA6.

Out of 336 beet samples from Poland analyzed by DAS-ELISA, 46 were found positive for beet poleroviruses. Among these samples, five gave a positive reaction with the BChV-specific primers, and 39 with the BMYV-specific primers (P0 gene). In France, out of 61 beet samples analyzed using multiplex RT-PCR, 11, 20, and 9 tested positive for BChV, BMYV, and both viruses (indicating mixed infection), respectively.

The ranges of pairwise nucleotide sequence identities in the CP gene of BChV and BMYV isolates were 92.8 to 100 %. To better understand the molecular relationships among the beet polerovirus isolates, phylogenetic analysis was performed. Phylogenetic trees for the CP gene were constructed using the K2 + G parameter. The results obtained using three different algorithms (NJ, ML and ME) were similar, and only the NJ tree is presented in Figure 1a. The 26 CP gene sequences, at the nucleotide level, can be divided into five clusters, three for BMYV isolates and two for BChV, named BM1 to BM3 and BC1 to BC2, respectively (Fig. 1a). The Polish BMYV isolates grouped in two separate clusters, BM2 and BM3, whereas the French BMYV isolates (8/9) clustered mainly in the phylogroup BM1 (Fig. 1a). Two separate phylogenetic trees were constructed for the P0 gene using BYMV and BChV sequences, respectively (Fig. 1b and c). A phylogenetic tree for BChV was constructed using the K2 parameter model, whereas for BYMV, we used K2 + G. BChV isolates displayed a high level of genetic similarity, and pairwise distance among them was 97.3 %-100 %, whereas BYMV isolates were more diverse and displayed 92.9 %-99 % nucleotide sequence identity.

The normalized $d_N - d_S$ value was calculated for each codon of CP and P0. For several of the CP codons, this value was less than one, indicating the operation of purifying (stabilizing) selection. A similar situation was observed for the P0 gene of BChV, whereas in BMYV, besides the negatively selected sites, episodic diversifying selection ($d_N - d_S$ value higher than one) was identified by all the methods used for codons 131, 181 and 228. Interestingly, the CP gene sequences of two French BChV isolates, designated as BChV-O36 and BChV-N13, fall within the group of BMYV isolates, suggesting possible recombination between members of these two species (Fig. 1a). High Z-values confirm the statistical evidence for recombination events (Fig. 2). The SBP and GARD methods indicated a single recombination breakpoint at approximately position 410 in the CP gene. It has been shown previously that the major recombination hotspots for these poleroviruses are localized within the intergenic (non-coding) region [16]. Recently, Schneider et al. [25] have shown evidence for recombination within the CP of the luteovirus soybean dwarf virus. Further research based on full-length genome sequences is required to establish other breakpoints. Interestingly, these two recombinant BChV isolates originated from two locations about 200 km apart from each other, in two different French departments, Nord and Oise.

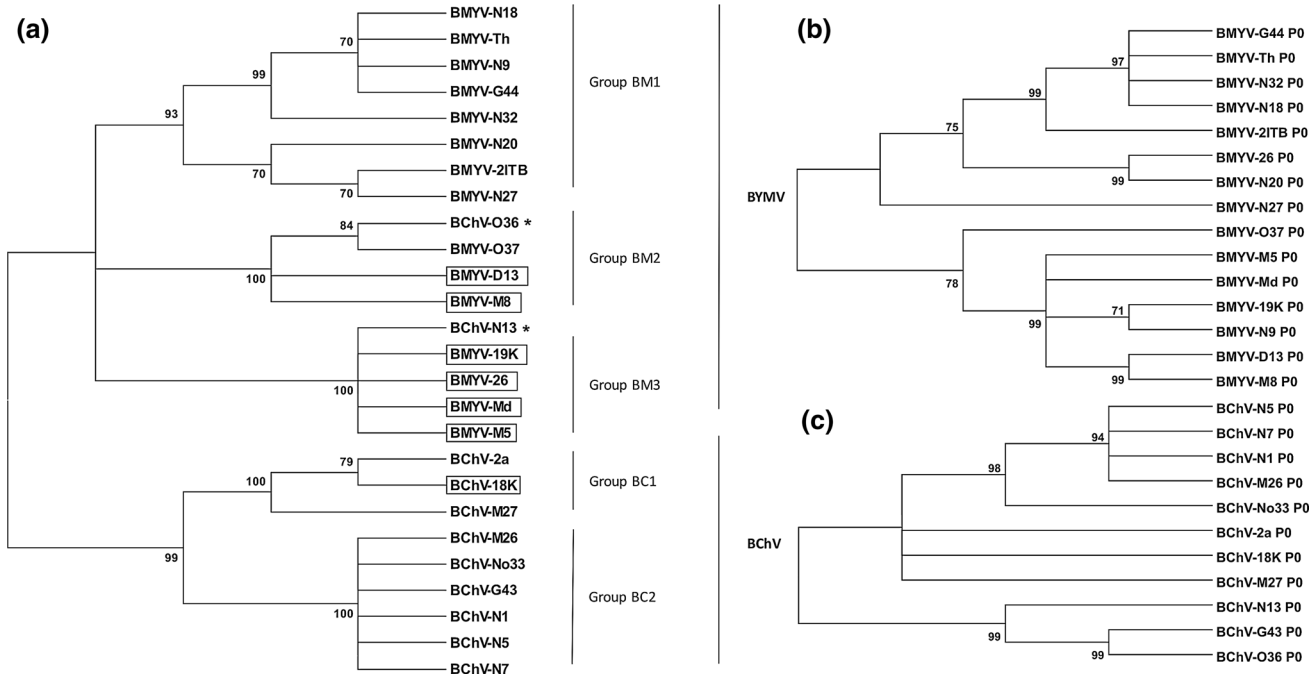

Fig. 1 Neighbor-joining trees constructed with MEGA6 software using (a) CP sequences (563 nucleotides) of 24 beet poleroviruses, (b) P0 of 14 isolates of BMYV (720 nucleotides), and (c) P0 sequences of 10 BChV isolates (747 nucleotides). Numbers on branches indicate the percentage of bootstrap support out of 1000 bootstrap replications (values below 70 % were collapsed). Polish isolates are shown in boxes, and recombinant isolates are marked with asterisks

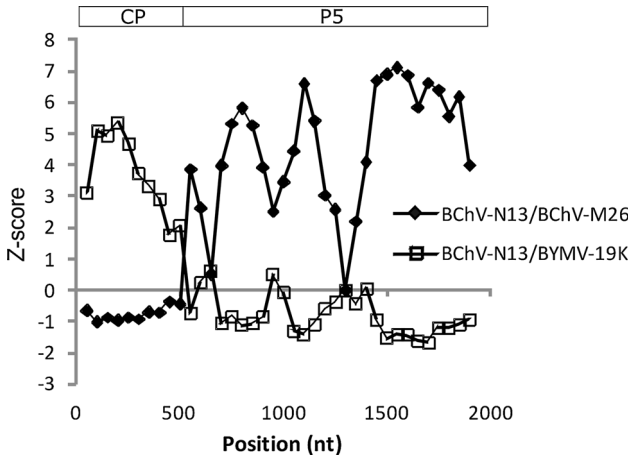

Fig. 2 SISCAN analysis of aligned nucleotide sequences of the RT protein (2004 nt) of the BChV- N13 isolate and its potential parents (CP of BMYV-19K and P5 of BChV-M26). The graph is based on Z-values using the total nucleotide identity scores

The main objective of this work was to investigate the molecular variability of the structural protein (CP) and the non-structural protein P0 genes in populations of beet poleroviruses sampled from two geographically distant European countries. Phylogenetic studies are thought to be the best tool for representation of viral population structure [17]. Our phylogeographic analysis of the CP gene demonstrates some relationships to the geographical origin of beet polerovirus isolates. Indeed, Polish BMYV isolates are grouped into the clusters BM2 and BM3, whereas the cluster BM1 is only composed of French isolates (Fig. 1a). Nevertheless, some isolates from distant regions have identical amino acid sequences, e.g., French and Polish isolates included in cluster BM3 displayed 100 % amino acid sequence identity. Therefore, these French and Polish variants could be considered members of the same BMYV population. Interestingly, the variability of beet polerovirus seems to be lower in Poland than in France.

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