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ORIGINAL ARTICLE

Molecular characterization and comparisons of potato wart (*Synchytrium endobioticum*) in historic collections to recent findings in Canada and the Netherlands

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Abstract

Synchytrium endobioticum (Schilb.) Perc. is a chytrid fungus causing potato wart disease and is one of the most important quarantine diseases on cultivated potato. Infected host tissues develop warts rendering the crop unmarketable. Resting spores, that can remain viable and infectious for decades, are formed in warted tissues and are released into the surrounding soil when host tissue decays.

To better understand the pathogen's diversity and to potentially uncover pathways of migrations and introduction events, molecular characterization was performed on the historical *S. endobioticum* resting spore collection of the Dutch National Plant Protection Organization. Mitochondrial genomes were assembled and annotated, and four novel structural variants were identified from these materials with intronic presence-absence variation in *cox1* or *cob* genes and structural variation in the *dpoB* – TIR region. Several fungal isolates were shown to contain mixtures of structural variants. We analyzed the mitogenomic sequences obtained from recent potato wart disease findings in Canada and the Netherlands in the context of the historical materials and found that fungal isolates from the new Dutch outbreak contained a specific mixture of mitogenomic variants previously not observed in the Netherlands. Based on the mitogenomic profile, pathotype 38(Nevşehir) was suspected which was later verified with the Spieckermann bioassay. To further facilitate dissemination of data and interactive visual analytics we created a public Nextstrain webpage with *S. endobioticum* mitogenomic sequences and associated metadata on their geographic origin, pathotype identity and (mixture) of mitogenomic variants (https://nextstrain.nrcnvwa.nl/Sendo).

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Introduction

Collections of plant pests offer a unique opportunity to place disease outbreaks in a historical and geographic context. To achieve this, population genomics can be exploited to compare materials from novel outbreaks with those collected from previous outbreaks along with samples from the presumed center of origin. Such analyses are not only instrumental to understand the pathogen's diversity, but also might uncover pathways of migrations, hubs and introduction events. The quarantine fungus causing potato wart disease, *Synchytrium endobioticum* (Schilberszky) Percival, is a high profile candidate for such studies as cases are often well documented and findings are described in great detail. The pest was introduced in Europe from its original range in the Andean region in the late 1800's (Chisnall Hampson 1993) and has since then become established in countries in Europe, Africa, Oceania, Asia and North and South America. Spread of this obligate biotrophic chytrid is mainly attributed to human activity and the transport of infected seed potatoes, soil, and fertilizers were implicated as important factors for local and long-distance dispersal (van de Vossenberg et al. 2022; Gough 1919). Furthermore, disease incidence is relatively low with an average of two new findings reported per year worldwide over the last two decades (EPPO global database, https://gd.eppo.int/taxon/SYNCEN/reporting, last accessed 1 August 2022). Often material from these outbreaks is preserved in collections and available for molecular and genomic studies.

For *S. endobioticum*, the 72.9 kb mitochondrial genome proved to be a suitable population genomic marker (van de Vossenberg et al. 2018). The linear mitochondrial genome contains fourteen protein coding genes typically found in fungal mitogenomes, Terminal Inverted Repeats (TIRs), a reduced set of tRNAs corresponding with the Chlorophycean Mitochondrial Code (translation Table 16), a DNA polymerase B (*dpoB*) homolog and several intron encoded homing endonuclease genes (HEGs) in the cytochrome oxidase c subunit 1 (*cox1*) and cytochrome b (*cob*) genes. A population genomic study with 30 fungal isolates, showed that *S. endobioticum* isolates shared a syntenic and largely similar mitogenomic structure. Sequence diversity of these 30 isolates resulted in clustering of the isolates in four main clusters in a haplotype network analysis, and it was hypothesized the pathogen was introduced in Europe multiple times independently (van de Vossenberg et al. 2018).

Here we report an extended evaluation of mitogenomic diversity of isolates from the historical *S. endobioticum* collection from the Dutch National Plant Protection Organization (NPPO-NL). This collection consists of containers with a mixture of resting spores in sandy soil. It contains materials from Dutch outbreak sites since the 1980's and *S. endobioticum* samples provided to NPPO-NL by other countries for molecular characterization and phenotypic characterization for virulence on a differential set of potato cultivars (pathotyping). From these sequences, novel mitogenomic structural variants were determined and several fungal isolates were shown to contain mixtures of structural variants. We further demonstrated the use of the mitogenomic diversity by analyzing sequences obtained from recent potato wart disease findings in Canada (2015–2017) and the Netherlands (2020) in the context of the historical accessions.

For a quarantine pest such as *S. endobioticum*, up-to-date and interactive analysis can greatly add in the interpretation of complex migration pathways that are often associated with plant pathogens. To further exploit the population genomic characterization and facilitate the dissemination of the results on diversity and migration to end-users, we created a public Nextstrain webpage to share and visualize *S. endobioticum* mitogenomic sequences in the context of geographic origin, pathotype identity and (mixture) of mitogenomic variants. Nextstrain is a bioinformatic toolbox initially build for the analysis and dissemination of information on viral pathogens to humans (Hadfield et al. 2018). Apart from human viral pathogen, Nextstrain builds have been created for several plant pathogens including the fungus *Puccinia striiformis* f. sp. *tritici* causing wheat yellow rust disease (Adams et al. 2020) and tomato brown rugose fruit virus (van de Vossenberg et al. 2020b). The *S. endobioticum* Nextstrain build is available via https://nextstrain.nrcnvwa.nl/Sendo/ (last accessed 1 August 2022).

Materials and methods

NPPO-NL collection The NPPO-NL *S. endobioticum* collection is made up of approximately 200 containers of sand mixed with resting spores dating back to 1986 and originating from outbreaks in Europe, North America and Asia. The collection samples are primarily used as inoculum for bioassays and are also referred to as composts. Collection materials were prepared by cutting potato warts obtained from either field sampling or bioassays into small (~ 1 cm) pieces and mixing them with clean sand in a 1:3 wart to sand volume ratio. Mixtures were incubated for four to six months at 15 to 20 °C and moistened with tap water two to three times per week for the first two months. Mixtures were stirred two to three times per week for the first two months and weekly for the next two months. After four months, the mixtures were no longer stirred or moistened but slowly air dried. Once completely dried, material was transferred to containers for long term storage at ambient temperature (~ 18 °C). A systematic naming system was introduced to allow easy

identification of collection samples. This consisted of a SeTr (after the Euphresco SendoTrack project) prefix identifier for isolates (e.g. SeTr-048 for isolate MB2) and a sequential follow-up number (-001, -002, etc.) to represent biological replicates or derived materials. This naming system provides unique identifiers and facilitates correct identification of individual collection accessions and how they are related (e.g. SeTr-048-001 to SeTr-048-015). Accessions of isolates that were already sequenced, that could be linked to a SeTr identifier, but could not be linked to an individual collection accession were given a '-000' as the sequential number. An overview of materials that were included in the Nextstrain build, SeTr-identifiers, other identifiers (when available) and associated metadata is presented in S-Table 1.

Zonal centrifuge and DNA extraction Per container with inoculum, 200 g subsamples were taken and used for resting spore extraction with a zonal centrifuge following the methodology described by (Wander et al. 2007). In short, the subsample is mixed with tap water up to 1 L and 1 mL of 1% Triton X-100 and the following reagents are automatically added to the Hendrickx centrifuge rotor, which rotates horizontally at 15,000 *xg*: (1) 30 mL $CaCl_2$ solution (specific gravity 1.4); (2) additional tap water; (3) 500 mL inoculum suspension, and (4) 60 mL kaolin suspension (200 $g/L_{tap\ water}$). The $CaCl_2$ solution containing resting spores was collected in a small beaker and subjected to filtration over a 20 µm nylon net sieve. DNA and RNA was extracted from isolated spore preparations, using the Allprep PowerFecall kit (Qiagen) according to the protocol of the manufacturer. Resting spore suspensions (approximately 100 µL) were collected in a 2 ml bead beat tube containing pre warmed (55 °C) lysis buffer PM1, DTT and 3 stainless steel balls 3.2 mm. The lysis was processed in Precellys Evolution Homogenizer (Bertin, France) instruments for 3 × 20 s at 5,800 bpm. DNA was eluted in 60 µL molecular grade water.

Sampling and DNA extraction of Canadian outbreak samples Three plots were surveyed as part of ongoing in-field variety wart susceptibility trials from 2015 to 2017. Tubers displaying wart symptoms were collected and packaged in plastic-lined paper sampling bags according to host variety. DNA was extracted from isolated spore preparations. Spores (approximately 80 mg) prepared from tuber warts were collected in a 2 mL tube and resuspended in 0.5 mL lysis buffer KF (Promega, WI, USA) with 2 µL RNAse A (110 mg/ml), 25 µL antifoam B (Sigma, MA, USA), and 1.5 g of 2.0 mm zirconia beads were added. The mixture was processed in a Beadbeater 96 (Biospec, OK, USA) for 1 min at room temperature to lyse the spores. After centrifugation for 1 min at 10,000 *xg* the supernatant was transferred into a clean tube. The pellet was resuspended with another 0.5 mL of lysis buffer KF, incubated at 65 °C for 10 min and centrifuged again for 1 min at 10,000 *xg*. The supernatant was added to the first supernatant, and 100 µL of 10 M ammonium acetate was added, mixed, and incubated at 4 °C for 30 min. DNA was recovered from the lysate using the Genomic KF kit (Promega, WI, USA) on a Kingfisher magnetic particle processor (ThermoFisher, MA, USA). DNA was eluted in 125 µL molecular grade water and was used immediately or stored at -20 °C until use.

Sampling and DNA extraction of Dutch outbreak samples During an official *S. endobioticum* survey in October 2020, warted tubers were found on the starch potato variety Festien in three fields (total of 14.43 ha) in the municipality of Stadskanaal: one field in Mussel (SeTr-136) and two fields in Onstwedde (SeTr-137 and SeTr-138). Following the initial finding, additional warted tubers and tare soil were collected. Warts (5–10 pieces) were taken from infested tubers and transferred to a GrindoMix beaker with cutting knife (Retsch, Germany) and 100 mL tap water. Warts were grinded for 20 s at 10,000 rpm in a GM200 GrindoMix (Retsch, Germany) and the homogenized material was processed using two stacked sieves with mesh sizes of 75 µm for the upper sieve, and 25 µm for the lower sieve. Resting spores were collected from the 25 µm sieve and used as input for DNA extraction as described for compost samples. For the extraction of spores from tare soil, sieving method B described in the *S. endobioticum* EPPO standard PM7/28 (2) (EPPO/OEPP 2017) was used. The resulting spore suspension in saturated $CaCl_2$ solution was subjected to filtration and DNA extraction as described above.

Pathotyping outbreak samples The Canadian outbreak samples were pathotyped following the Glynne-Lemmerzahl bioassay as described in EPPO standard PM7/28 (2) (EPPO/OEPP 2017). Green warts were propagated on variety Arran Victory (isolates SeTr-139 and SeTr-140) or Deodara (SeTr-141) following inoculation with the original field material. Pathotyping was performed with potato varieties Deodara, Producent, Saphir, Delcora, Miriam, and Ulme. At least 16 tubers per differential cultivar were used, and reaction types were scored after 25 to 30 days.

The Dutch outbreak samples obtained from the three fields were pathotyped using the Spieckermann bioassay as described in EPPO standard PM7/28 (1) (EPPO/OEPP 2004). Wart material was composted prior to inoculation on potato varieties Deodara, Producent, Talent, Saphir, Belita, and Karolin. Per variety, approximately 54 tuber blocks were inoculated and reaction types were scored 8 to 10 weeks after inoculation. Apart from the Dutch outbreak samples, two control samples were included in the Spieckermann bioassay, namely a pathotype 18(T1) compost (HLB05-08) and a Turkish compost (SeTr-009-002) with presumed pathotype 38(Nevşehir) identity.

Pathogen quantification*S. endobioticum* DNA obtained from the collection accessions was quantified using either a *S. endobioticum* specific real-time PCR described by Van Gent-Pelzer et al. (2009) or one described by Smith et al. (2014). Reaction mixes were based on the Premix Ex Taq Master Mix (Takara Bio, Japan) or Maxima qPCR master mix (Thermo Fisher, MA, USA) reagents respectively. Apart from the *S. endobioticum* specific real-time PCR tests, a generic plant *cox1* real-time PCR test (Mumford et al. 2004) was performed in a separate reaction. Reactions were performed in a CFX96 touch (BioRad, CA, USA) using thermocycler program 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Alternatively reactions were performed in a Quantstudio 12 K flex (Thermo Fisher, MA, USA). DNA extracts with exponential amplification curves and Cq values below 30 for the species specific assay were selected for Illumina whole genome shotgun sequencing.

Illumina sequencing Prior to library preparation, genomic DNA extracts were quantified using the Qubit (Thermo Fisher, MA, USA) with the broad range reagents for double stranded DNA. Library preps were 150 paired-end sequenced with one or more of the following Illumina (CA, USA) platforms: MiSeq, HiSeq 2500, NextSeq 500, NextSeq 2000, or NovaSeq 6000 System. Additional details are provided in S-Table 1. Sequence read archives (SRAs) were deposited on the European Nucleotide Archive under accessions ERR10640993 to ERR10641135.

Assembly of mitochondrial genomes Illumina reads were mapped to the pathotype 1(D1) MB42 reference mitogenome (NCBI GenBank: MK292680) using the "Map reads to reference" tool (length fraction: 0.8, similarity fraction: 0.9) in CLC genomics workbench v21.0.3 (Qiagen, Germany). Each read mapping was visually checked to identify potential issues (S-Fig. 1). Four additional mtDNA variants with structural variant in either *cox1*, *cob* or the *dpoB*-TIR region were identified. For each of these variants, a reference guided *de novo* assembly was performed with GRAbB in combination with the SPAdes assembler (Brankovics et al. 2016). References used in the GRAbB assemblies consisted of reliable consensus sequences flanking the problematic areas. The resulting *de novo* assemblies were verified with read mapping in CLC genomics workbench (length fraction: 0.8, similarity fraction: 0.9) and were improved with Pilon v1.24 (Walker et al. 2014). For each variant, a structural and functional annotation of mitogenomic elements was performed with Mitos v1 (translation table: 16) (Bernt et al. 2013) which was implemented as an external application in CLC genomics workbench (https://github.com/NPPO-NL/Mitogenome-and-rDNA-assembly-and-annotation-pipeline/tree/main/CEAs/CEA009_mitos_annotation, last accessed 1 August 2022). Predicted protein coding gene annotations were visually inspected to determine if start and stop codons were predicted correctly. When introns were observed in mitogenomic protein coding genes, open reading frames (ORFs) were identified with the "Find ORFs" tool (translation table: 16) incorporated in Geneious Prime v2022.0.1 (Biomatters, New Zealand). Functional annotation of the predicted ORFs were verified by analyzing the derived amino acid sequences in InterProScan v5.56-89.0 (Blum et al. 2021). Again all available datasets (including public sequence read archives) were mapped to the five main mitogenomic variants (alpha, beta, gamma, delta, epsilon) in CLC genomics workbench (length fraction: 0.8, similarity fraction: 0.9), mappings were visually assessed to identify potential issues in the assembly and mapping of reads, and consensus sequences were extracted (low coverage definition: 10, low coverage handling: remove low coverage and split sequence, conflict resolution: use quality scores, consensus sequence decoration: transfer annotations from reference). The complete mitogenomic sequences were submitted to NCBI GenBank under accessions OP142534 to OP142675.

Haplogroups and mixtures of structural variants The obtained complete *S. endobioticum* mitogenomes were aligned with the *Synchytrium microbalum* mitogenome (NC_042878) using MAFFT v7.45 (Katoh and Standley 2013). Intronic sequences were removed prior to alignment. ModelTest was performed in CLC genomics workbench and a Bayesian inference of phylogeny was performed with the optimal substitution model (GTR + G + I) with the MrBayes v3.2.6 plugin incorporated in Geneious (10^6 generations, subsampling every 200 generations, 10% burn-in). Trees were combined to generate a 50% majority rule consensus tree with posterior probabilities which was rooted using the *S. microbalum* mitogenome.

Population level relationships of the *S. endobioticum* mitogenomes (introns excluded) were determined by creating a median joining (MJ) network (Bandelt et al. 1999) with POPart (Epsilon = 0) (Leigh and Bryant 2015). Haplogroups were defined as groups of sequences that were supported by both the Bayesian and MJ network clustering. Singletons and sequences that did not group similarly in both clustering methods were defined as 'unplaced'.

To determine if multiple different structural variants were present in a single sample, reads were mapped to variant specific sequences in CLC Genomics workbench (length fraction: 0.8, similarity fraction: 0.9). For variant α the *dopB* – TIR region was used (SeTr-060-000: position 64,871–66,449). This region is present in all variants except in variant ε. For variants β and γ the variant specific *cox1* introns (SeTr-085-003: 34,052–35,386 and SeTr-135-001: 34,701–37,252 respectively) were used, and for variants δ and ε the variant specific *cob* intron (SeTr-117-001: 49,044–51,711)

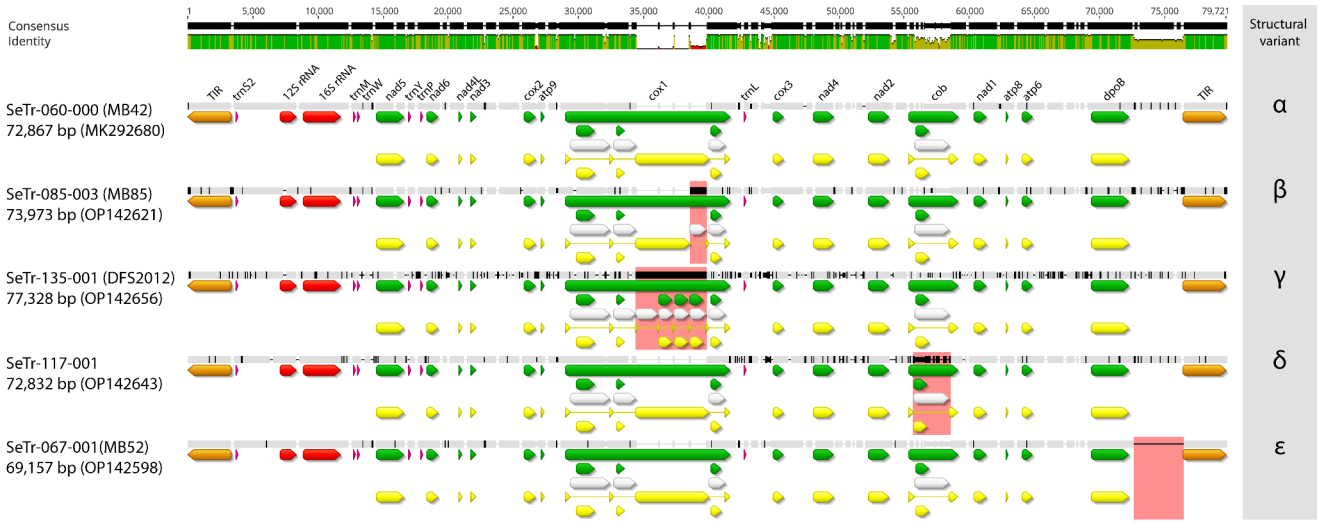

Fig. 1 MAFFT alignment of *S. endobioticum* mitogenomic structural variants. Per structural variant a single representative sequence is shown. Annotation labels are provided for structural variant α (excluding the intronic open reading frames = iORFs). Differences relative to the overall consensus sequence in the 79,721 nt (including gaps) alignment are highlighted in black. Structural differences relative to variant α are indicated with red boxes, i.e. β = single additional intron with iORF in *cox1*; γ = four additional introns and three iORFs in *cox1*; δ = *cob* gene with another intronic sequence and iORF; ε = deletion of *dpoB* – TIR region

and *dpoB* – TIR region (SeTr-067-001: 65,267 – 66,143) were used respectively. A variant other than α was considered present when paired reads covered the entire variant specific sequence.

Nextstrain implementation To identify potential links between genotypes and geographical origin or pathotype identity, an interactive Nextstrain build was created based on the complete mitogenomic sequences of the 172 *S. endobioticum* isolates. Augur (github.com/Nextstrain/augur), a bioinformatics toolkit for phylogenetic analysis used in the Nextstrain pipeline, was used to align the mitogenomic sequences with MAFFT (Katoh and Standley 2013) and to build a RAxML maximum likelihood phylogeny (Stamatakis 2014). The initial tree was refined with metadata and a time tree was build using TreeTime (Sagulenko et al. 2018) with optimization of scalar coalescent time and using a fixed clock rate for early diverging fungi of 1.51×10^{-9} per year (Aguileta et al. 2014). Internal nodes were assigned to their marginally most likely dates, and confidence intervals for node dates were estimated. Amino acid changes were determined relative to the MB42 reference mitogenome (MK292680). Augur output was exported and visualized in Auspice.

Results

Sampling and sequencing statistics Of the approximately 200 accessions in the potato wart collection and samples from recent findings subjected to spore extraction, 150 produced spore extracts with real-time PCR Cq values ranging from 12.7 to 34.6 ($mean_{Cq}$ = 22.4, StDev = 3.3) which were Illumina sequenced. Sequence yield of these samples ranged from 1.7 to 24.7 gigabases ($mean_{yield}$ = 9.7 Gb, StDev = 4.3 Gb). On average 3.7% (StDev = 5.5%, range = 0.07–32.5%) of all data generated for a sample mapped to the mitogenome. In 68% of the samples sequenced in this study an average read coverage (ARC) of the mitogenome between 1,000 and 7,500x was obtained. Twenty-seven samples had an ARC between 78 and 1,000x, and thirteen samples had ARCs between 9,000 and 40,000x. In eight cases the ARC was lower than 50x and the obtained data could not be used to reliably assemble the mitogenome resulting in a total of 142 novel mitogenomes assembled and annotated in this study.

Detection and characterization of structural variants Read mappings to the MB42 reference mitogenome (MK292680) revealed conflicts in several isolates in either the intron-containing *cox1* or *cob* gene or the *dpoB*-TIR region resulting in the identification of five structural variants. The GRAbB assemblies were able to resolve these conflicts (S-Fig. 1) revealing a dynamic intronic content for the *cox1* and *cob* genes and partial absence of the *dpoB*-TIR region in some isolates (Fig. 1). Relative to the Dutch pathotype 1(D1) reference isolate MB42 (with a mitogenomic architecture hereafter referred to as structural variant α), the Greek pathotype 18(T1) isolate SeTr-085-003 isolate has a forth intron in the mitochondrial *cox1* gene (variant β). No HEG could be identified in this intronic sequence. The Scottish pathotype 1(D1) isolate SeTr-135-001 (variant γ) was found to contain seven introns and six intron encoded HEGs. The German pathotype 2(G1) isolate SeTr-117-001

(variant δ) contained a similar intron count as the MB42 variant α sequence, however the *cob* gene was invaded by a different intronic sequence relative to the other variants. The intronic sequence of variant δ had a pairwise sequence identity of 53.4 to 54.5% to the intronic sequence of the other *S. endobioticum* mtDNA structural variants (S-Fig. 2), whereas the intronic sequence shared by variants α, β, γ and ε were highly similar: 97.1 to 100%. Furthermore, the intronic sequence encoding a LAGLIDADG HEG in SeTr-117-001 was spliced into the *cob* gene 50 bases upstream relative to the other variants underlining the invasion by a different HEG with another recognition site. The Polish pathotype 2(Ch1) isolate SeTr-067-001 (variant ε) showed partial absence of the *dpoB*-TIR region. No amino acid sequence differences as a result of intronic content variation was observed, i.e. amino acid sequences for all *cox1* variants were 100% identical, and for the cob 99.7 to 100% identical amino acid sequences were obtained (S-Fig. 3).

Haplogroups Analysis of the intron excluded mitogenomic sequences with outgroup resulted in a 66,751 nt alignment (mean ungapped length 64,517 nt, 53 parsimony informative sites) that was used to construct a phylogeny using Bayesian inference, and a 66,045 nt alignment for haplogrouping without outgroup (mean ungapped length 64,754 nt, 267 parsimony informative sites). Of the 172 sequences, 166 sequences clustered in one of six haplogroups that were supported by the Bayesian tree (Fig. 2) and the MJ haplotype network (Fig. 3). Pathotype 18(T1), a pathotype of economic importance, is only found in haplogroup 3. Other pathotypes of major economic importance, i.e. 1(D1), 2(G1), 6(O1), are found in various haplogroups (Table 1).

Detection and quantification of mixtures of structural variants In the majority of samples analyzed ($N = 116$, 67.4%) only a single mitogenomic variant was detected (S-Table 1). These were either variant α, β or δ. Variants γ and ε were only found in combination with another structural variant. Samples in which two mitogenomic variants were observed represented 30.2% ($N = 52$) of the sample set. Twenty-two of those represent a combination of β and δ mitogenomes found in pathotype 38(Nevşehir) samples from haplogroups 3 (in samples where variant β is most prominent) and 5 (in samples where variant δ is most prominent). Peruvian sample PER03 contained three mitogenomic variants (α, β and γ), and in three samples (SeTr-132-001, SeTr-133-001, SeTr-134-001) from the same locality an impressive four mitogenomic variants (α, β, γ and δ) were found to co-occur.

Nextstrain build The *S. endobioticum* Nextstrain build contains complete mitogenomes (introns excluded) of 172 *S. endobioticum* isolates generated from material sampled between 1986 and December 2020, originating from 16 countries. Information in the build is presented in three main panels: clustering of genomic diversity, geographical origin of the samples, and diversity relative to MB42 pathotype 1(D1) mitogenome MK292680 (Fig. 4). Users can color the external nodes in the phylogenetic tree based on pathotype, haplogroup, mtDNA structural variant(s) and origin. Branch lengths of the tree can be shown based on divergence or in function of time. Nextstrain determines the most likely transmission events based on the information included in the build, which can be animated from the webpage. The genotypes represented in the tree are plotted on a map and users can set different levels of geographical resolution, i.e. continent, country, state and municipality (when this information is available). This, together with the use of filters, allows flexible simultaneous interrogation of phylogenetic and geographic relationships in the context of epidemiological metadata.

Characterizing outbreak samples Canadian Newfoundland samples SeTr-139-001 and SeTr-140-001, from Carmanville and Saint Phillips respectively, both produced a pathotype 6(O1) phenotype. Potato varieties Deodara and Producent were very susceptible to the two isolates, whereas varieties Saphir, Miriam and Ulme showed no wart formation in the bioassay. For the variety Delcora, slightly different reactions were obtained with SeTr-139-001 and SeTr-140-001, resulting in a weakly resistant and in an extremely resistant interaction respectively. Isolate SeTr-141-001 from Avondale (Newfoundland) produced a pathotype 8(F1) phenotype with varieties Deodara, Producent, Delcora being very susceptible to the isolate, whereas Saphir, Miriam and Ulme were either resistant or extremely resistant to the isolate. All three samples produced a haplogroup 1 - structural variant α mitogenome similar to the seven historical Canadian isolates included in the analysis, which represent pathotype 2(G1) and 6(O1) isolates sampled between 1995 and 2014 from both Newfoundland and Prince Edward Island (PEI). Apart from the variant α mitogenomes, no other variants were found to co-occur in the Canadian samples. Within haplogroup 1 the new Canadian outbreak samples cluster with Canadian pathotype 6(O1) isolate LEV6574 and several Dutch pathotype 1(D1) isolates (SeTr-048, SeTr-056 and SeTr-061). When considering all Canadian isolates from Newfoundland and PEI sampled between 1995 and 2017, haplogroup 1 – structural variant α mitogenomes with 99.69 to 100% sequence similarity are observed. Within the Canadian sample set, the new Newfoundland isolates SeTr-139 to SeTr-141 are 99.99 to 100% similar, and the historical Newfoundland and PEI samples are 99.98 to 100% similar.

Inoculum produced from the Dutch 2020 outbreak samples SeTr-136 to SeTr-138, originating from Mussel and Onstwedde respectively, produced a pathotype 38(Nevşehir)

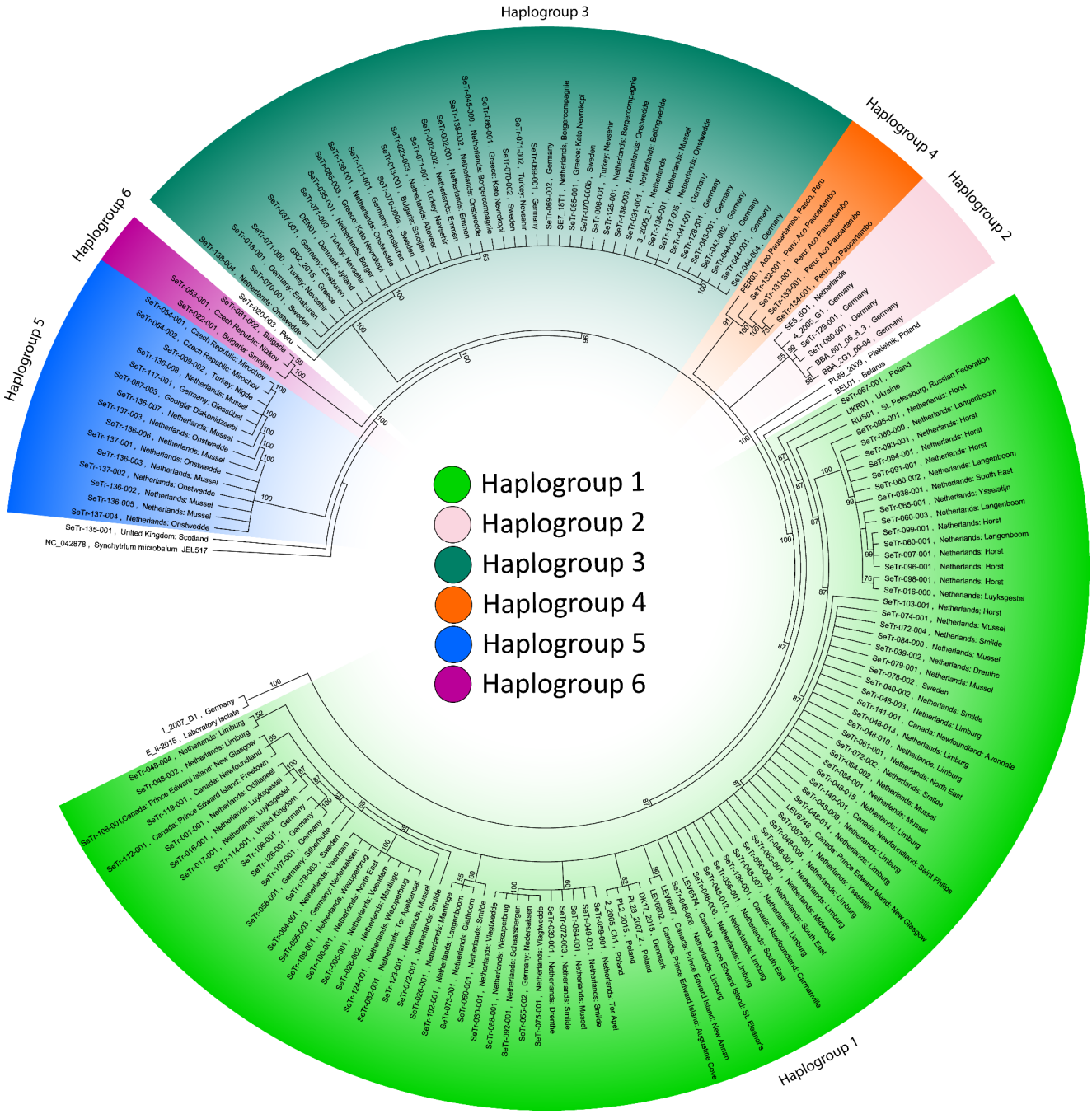

Fig. 2 Bayesian inference (BI) of phylogeny of intron excluded *S. endobioticum* mitogenomes. A 66,751 nt alignment with 53 parsimony informative sites was used to construct the tree under the GTR model with G + I distributed sites. The 23,811 bp circular *Synchytrium microbalum* mitogenome (NC_042878) served as outgroup to the *S. endobioticum* sequences. Bayesian posterior probabilities are displayed at branch nodes and mitogenomic haplogroups are highlighted in the tree. Haplogroups were defined as isolates grouping similarly in the Bayesian tree and the haplotype network. Haplogroup names for groups 1 to 4 were assigned as previously defined by Van de Vossenberg et al. (2018)

phenotype with potato varieties Deodara, Producent, Talent and Saphir being very susceptible, and varieties Belita and Karolin being resistant to the isolates. The pathotype 18(T1) control sample produced phenotypic results as expected. The suspected pathotype 38(Nevşehir) sample SeTr-009-002 did produce warts on all varieties where wart formation was expected, but the number of positive blocks on Saphir was very low compared to the Dutch outbreak samples. (S-Table 2). Microsatellite analysis of the warted tubers blocks verified the variety identity of Saphir in the

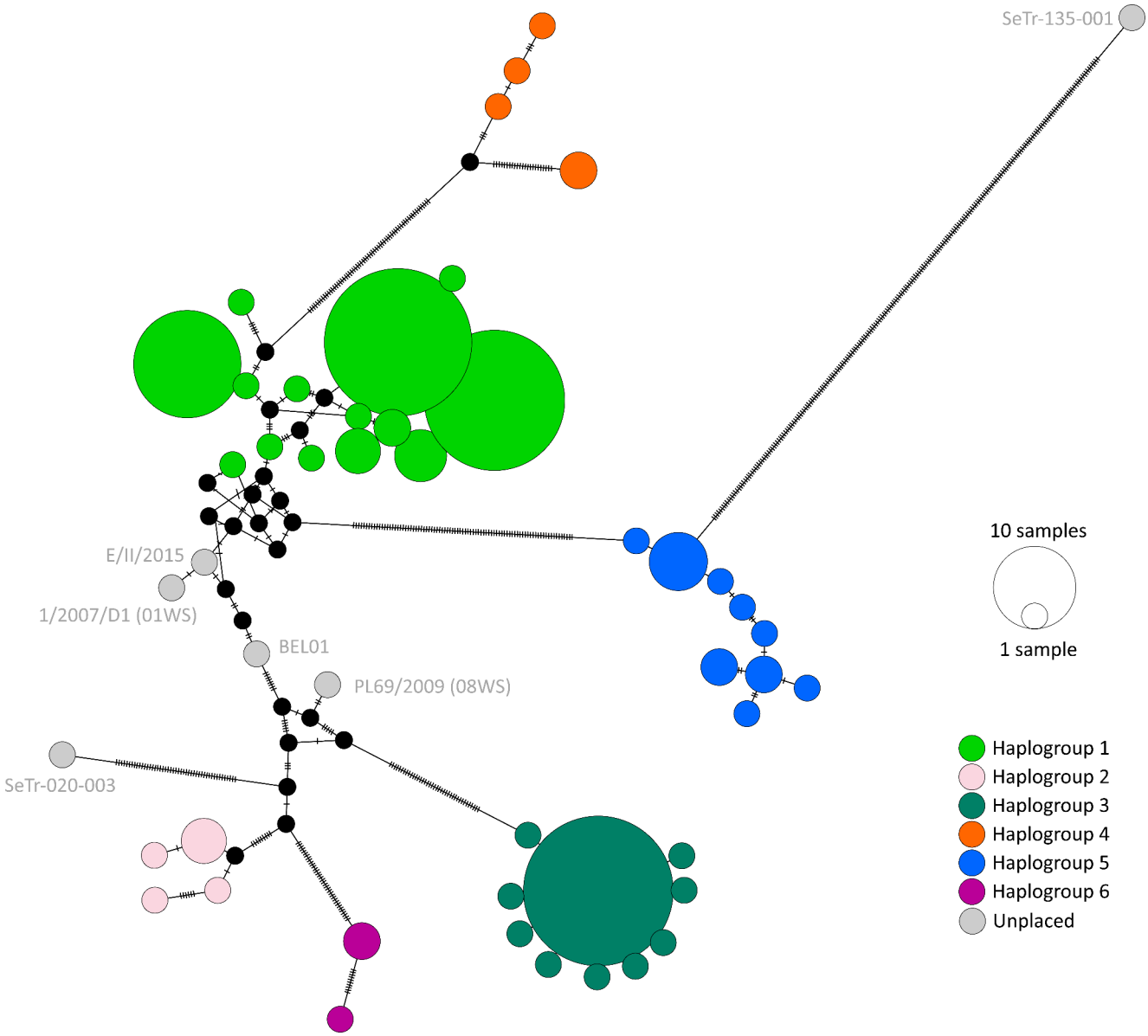

Fig. 3 Median Joining haplotype network of intron excluded *S. endobioticum* mitogenomes. The haplotype network was build based on 172 sequences resulting in a 66,045 nt alignment with 267 parsimony informative sites. Black nodes represent hypothetical ancestors and marks on the branches indicate the number of mutations. Colors are used to indicate the main haplogroups and unplaced sequences are indicated in grey. Haplogroups were defined as isolates grouping similarly in the Bayesian tree and the haplotype network. Haplogroup names for groups 1 to 4 were assigned as previously defined by Van de Vossenberg et al. (2018)

Table 1 Mitogenomic haplogroups and associations with mtDNA structural variants and pathotypes

Haplogroup	*N*	(combinations of) structural variants	Pathotypes	Countries
1	95	α (79), αβ (4) αδ (11), αε (1)	1(D1), 2(Ch1), 2(G1), 6(O1), 8(F1), 40(BN1), 41(P2)	Canada, Denmark, Germany, Netherlands, Poland, Russia, Sweden, Ukraine, United Kingdom
2	6	α (3), αδ (2), αε (1)	2(G1), 6(O1)	Germany, Netherlands
3	42	β (24), βδ (11), βε (7)	2(G1), 8(F1), 18(T1), 38(Nevşehir)	Bulgaria, Denmark, Germany, Greece, Netherlands, Sweden, Turkey
4	5	αδ (1), αβγ (1), αβγδ (3)	Pathotype identity not known	Peru
5	15	δ (4), βδ (11)	2(G1), 17(M2), 38(Nevşehir)	Czech Republic, Georgia, Germany, Netherlands, Turkey
6	3	α (3)	8(F1), 16(N1)	Bulgaria, Czech Republic
unplaced	6	α (3), βγ (1), αε (2)	1(D1), 6(O1), 39(P1),	Belarus, Germany, Peru, Poland, United Kingdom

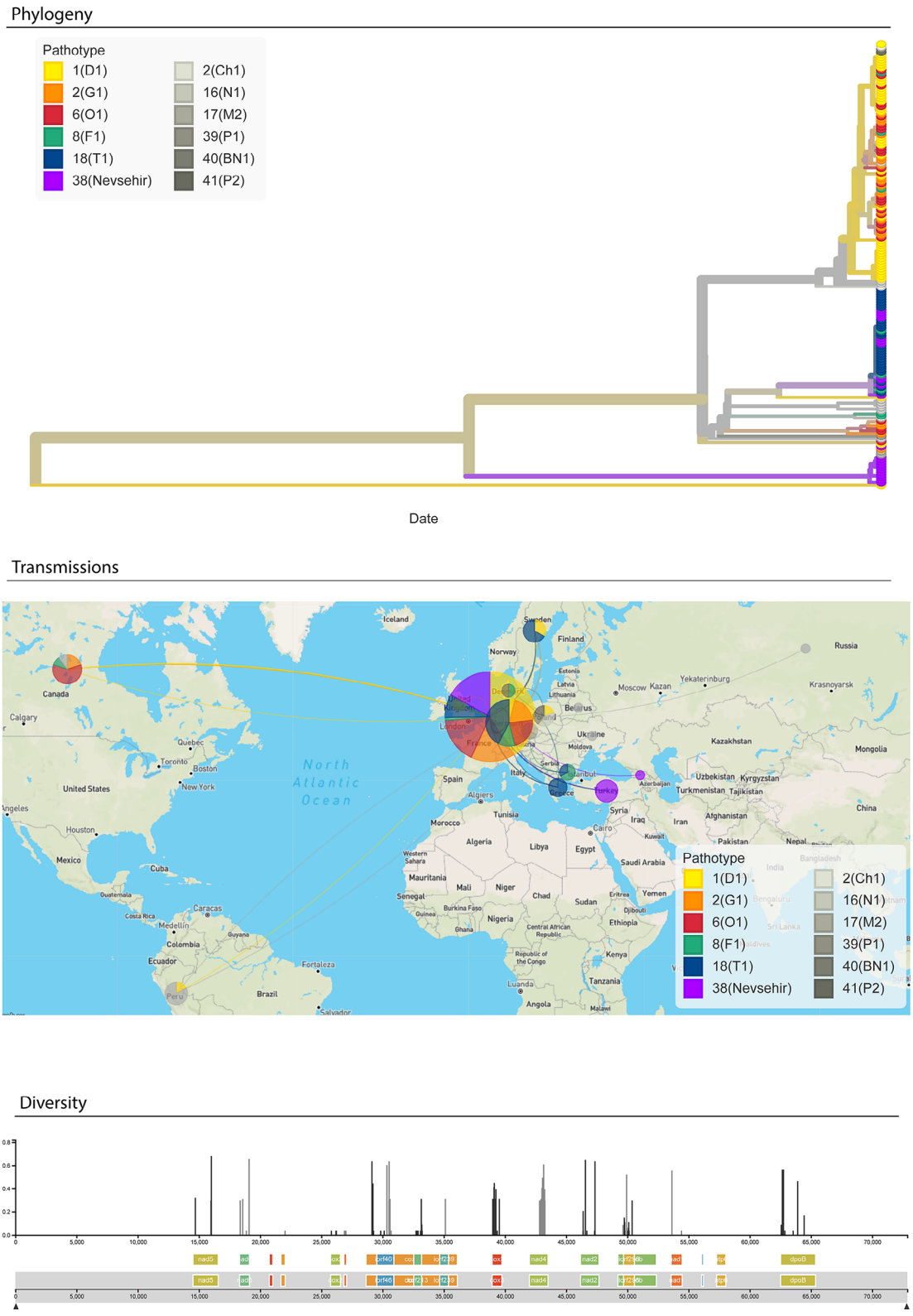

Fig. 4 Main panels of the *S. endobioticum* version 1 (20,220,801) Nextstrain build. Genomic epidemiology of 172 complete mitogenomes of materials sampled between January 1986 and December 2020 represented as a time tree (top panel) and the geographic distribution (middle panel) and diversity relative to the MB42 mitogenome MK292680 (lower panel). Users can use different features (e.g. pathotype, haplogroup, (mixture of) structural variant(s) and country) to color external nodes. Filters can be used to highlight intersections and subsets of the data. The *S. endobioticum* Nextstrain build can be accessed via https://nextstrain.nrcnvwa.nl/Sendo

SeTr-009-002 bioassay (data not shown) indicating that this isolate was indeed able to infect Saphir, which is not the case for pathotype 18(T1) isolates.

For samples SeTr-136 and SeTr-137 a total of 12 random subsamples were sequenced. All these subsamples generated sufficient coverage of the mitochondrial genomes to study both the haplogroup SNP's and structural variation as well as the possible ratio's between the haplogroup SNP's and structural variants identified in each sample. The combination of the haplogroups/structural variants was always haplogroup 5 – structural variant δ and haplogroup 3 – structural variant β mitogenome. Surprisingly the ratio between these haplotypes variants were remarkably different. Of the twelve, a haplogroup 5 – structural variant δ mitogenomic sequence was identified as most prominent haplotype (58.3–99.9% of average read coverage) in ten samples. In contrast, in two subsamples (one for each sample), a haplogroup 3 – structural variant β mitogenome was found to be most prevalent (75.3 and 88.1%). Presence of both variants β and δ was observed in sequences generated from both warted tubers and tare soil. Also, in the four subsamples obtained from the second Onstwedde field (SeTr-138), a mixture of mitogenomic variants β and δ was found. For these samples the haplogroup 3 – structural variant β mitogenome was most prominent with 80.6 to 94.2% of the average read coverage (S-Table 3). Other pathotype 38(Nevşehir) samples from the historical collection also contain a mixture of mitogenomic variants β and δ, varying from 0.1 to 94.2% for variant β.

Discussion

Understanding pathogen diversity at historical outbreak locations and in its native range are important to determine potential threats that come from new findings with altered population compositions such as increased genetic diversity. Sequencing of genomic regions offering population level resolution provide an opportunity to better understand pathogen diversity and spread. Having access to biological materials representing both outbreak sites and localities from its native range are a prerequisite for such studies. For *S. endobioticum* a large collection of inoculum is maintained at the Dutch NPPO with accessions dating back some 40 years. This is relatively long for a collection initially intended to preserve materials for future bioassays as resting spores in collection rarely remain viable beyond 10 years (van de Vossenberg et al. 2020a). With new sequencing techniques, new applications arise for the historical samples and old (non-viable) accessions become valuable again (Staats et al. 2013). The original intended use of the collection materials is also reflected in the metadata associated with the accessions. Quite often, date of production of the batch of inoculum, the country of origin and pathotype identity were recorded and preserved, but the precise locality, date of original sampling or potato varieties from which the materials were obtained is no longer available. With increased population level resolution and observed differences between isolates from the same country and pathotype identity, specific information on locality and potato varieties on which they were found or maintained become more important. Using the descriptors available from the collection accessions, we attempted to gather as precise metadata for the accessions as possible. Doing so we were able to correct some errors and our current overview represent to most comprehensive record of *S. endobioticum* accessions worldwide.

Mitogenomic sequences were assembled and annotated for the accessions in the collection allowing population-level analyses of new findings in a historical context. In general, fungal mitogenomic variation is the result of two types of events, namely small sequence variations (SNPs and short indels) and intron gain or loss (Brankovics et al. 2018). These introns can be extremely variable and differ even in genetically closely related isolates (Megarioti and Kouvelis 2020). In addition, these genes tend to move by horizontal transfer to new sites (Gonzalez et al. 1998). The intron lifecycle described for HEG-associated group I introns is characterized by introns with full-length HEGs, degenerate HEGs, introns lacking HEGs and intron absence (Haugen et al. 2005; Goddard and Burt 1999). In our previous study focusing on the *S. endobioticum* mitogenome (van de Vossenberg et al. 2018), presence-absence differences in intronic sequences were not observed. Assemblies of some isolates in the current study revealed conflicts in several *cox1* and *cob* introns and in the *dpoB* – TIR region which triggered an additional round of (reference guided) *de novo* assembly. This resulted in the identification of five different structural variants which were named α to ε.

Mitogenomic sequences obtained from recent Canadian and Dutch *S. endobioticum* findings were analyzed in the context of those from historical outbreaks and from Peru, the presumed region of origin of potato wart. The historical Canadian isolates sampled between 1995 and 2014 group in haplogroup 1 and are characterized by a structural variant α mitogenome. With the current methodology only a single structural variant could be detected. The historical samples include materials from PEI and Newfoundland (where the pest was first described in Canada in 1909 (Chisnall Hampson 1993)). The new Canadian findings all originate from Newfoundland and also group in haplogroup 1 and are characterized by a structural variant α mitogenome. From these observations, an initial introduction into Newfoundland and further spread to PEI, where it was first reported in 2000, is

a likely scenario. Variation observed between the Canadian isolates was extremely low and likely in the range of within field and within sample variation than to site-specific variation. Within-field mitogenomic variation is illustrated by isolate SeTr-048. In the fourteen pathotype 1(D1) subsamples taken from an infested field in Mill (the Netherlands) in 2001, a single mitogenomic variant was detected (variant α). The obtained consensus sequences clustered in haplogroup 1 and showed higher sequence diversity than the combined historical Canadian samples from Newfoundland and PEI.

Dutch historical samples are most abundant in our current analysis and are believed to represent most of the variation present in the Netherlands in the last decades. Intriguingly, analysis of mitogenomes obtained from new Dutch outbreak sites in the context of the historical collection revealed the presence of a (for the Netherlands) novel mixture of mitochondrial variants. The Dutch 2020 findings from Mussel and Onstwedde contained a specific combination of two structural variants (i.e. β and δ) currently only observed in pathotype 38(Nevşehir) isolates. These findings were instrumental in early communication with the potato growing sector regarding the potential of a (for the Netherlands) novel pathotype (i.e. 38(Nevşehir)) being present in the infested fields. It has to be noted that the mitogenome functions as a tracking marker and is not functionally associated with a pathotype identity. However for some mitogenomic variants there is a strong association with a single or a few pathotypes (e.g. isolates containing only variant β as observed in pathotype 18(T1) isolates). Early actions were taken and testing of resistance of popular starch potato varieties against the isolates from the novel outbreak sites were initiated. Pathotyping the isolates from the novel outbreak sites resulted in a pathotype 38(Nevşehir) phenotype six months after the mitogenomic analyses confirming the first finding of this pathotype in the Netherlands (Anonymous 2021).

Pathotype 38(Nevşehir) was previously reported from Bulgaria (van de Vossenberg et al. 2020a), Georgia (Sikharulidze et al. 2019) and Turkey (Çakır et al. 2009) and isolates from the latter two countries were included in our analysis. All sequenced pathotype 38(Nevşehir) isolates contain the specific combination of structural variants β and δ in highly varying compositions. Pathotype 38(Nevşehir) was first identified from Turkish material in 2006 (Çakır et al. 2009) and the use of field-resistant potato varieties was mentioned as a means of new pathotype formation. Under this model, selection against individuals carrying nuclear encoded virulence genes such as *AvrSen1* (van de Vossenberg et al. 2019) in the diverse genetic population could result in a selective sweep that could work either way while small amounts of the avirulent isolate are somehow retained in the population. Such effects have been observed for *Zymoseptoria tritici*, the fungus causing septoria leaf blotch in wheat, where mixed infections of virulent and avirulent strains allow the avirulent strain to be propagated albeit in this case in the sexual cycle (Barrett et al. 2021). Under this model the *S. endobioticum* pathotype 38(Nevşehir) phenotype could be the result of a mixture of an isolate containing only variant β (as observed in pathotype 18(T1) isolates) and an isolate containing only variant δ. The latter is rare and was only observed in a 2(G1) isolate from Germany (SeTr-117) and a 17(M2) isolate from the Czech Republic (SeTr-054). Mixing different pathogen populations could have occurred by introducing seed potatoes, compost or tare soil infested with one population into a field where another population was already present. Another potential source could be waste (tare soil or compost) from starch potato industries. When tubers from different sources infected with different populations of the pathogen are processed simultaneously or when waste is stored at a central location, mixtures with an additive effect could be created. If an added effect is obtained from mixed *S. endobioticum* populations, e.g. by escaping detection by current resistant potato varieties, extra caution should be taken and the use of tare soil and organic waste (composts) from potato processing industries on or near fields intended for potato cultivation should be prevented.

The presumed origin of the pest is only limited represented in the current study as just a few Peruvian samples could be included. However, these isolates contain mitogenomic sequences that are not represented at any of the other localities included in this study. As genomic variation is expected to be highest in the center of origin, this further strengthens the hypothesis of the Andean region being the center of origin for potato wart disease. Additional sampling and sequencing of materials from the presumed region of origin is highly recommended and remains to be done. Also, to further investigate the potential role of the United Kingdom as hub to the European main land in the late 1800's (Chisnall Hampson 1993), an attempt could be made to obtain and analyze historical English herbarium samples. This could prove to be very challenging as materials from those very old outbreaks are rarely available. Occasionally warted tubers are preserved in symptom flasks for educational purposes but usually no to limited information is available on the material preserved. Also, the usability of these preserved materials for molecular studies remains to be determined.

Finally, the prediction of transmission links and source attribution of the *S. endobioticum* Nextstrain build will improve with the inclusion of additional mitogenomes and metadata. Therefore, we encourage other organizations to share sequence data or biological materials with relevant metadata from historic materials and materials from new outbreaks to improve the build and our understanding of the

spread and diversity of this important fungal pathogen. The *S. endobioticum* Nextstrain build will be maintained and expanded by NPPO-NL.

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Author contributions BvdV and TvdL designed the study; JM, CH, KR-P, GvL, DS, KF and JP performed bioassays on Dutch, Canadian, German and Polish isolates; MvG, NtB, DS and JP optimized and performed molecular experiments; BvdV, TvK and HN performed bio-informatic analyses; DS, KF, JP, WP, EC and ZS collected and provided additional biological resources and associated metadata; BvdV wrote the first draft of the manuscript which was reviewed by TvdL, HN and GvL. All authors were included in preparation of the final version of the manuscript.

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Data availability The interactive *S. endobioticum* Nextstrain build webpage is publicly available via https://nextstrain.nrcnvwa.nl/Sendo. Sequence read archives (SRAs) were deposited on the European Nucleotide Archive under accessions ERR10640993 to ERR10641135. Structurally and functionally annotated mitogenomic sequences were submitted in NCBI under accessions OP142534 to OP142675.

Code availability The *S. endobioticum* Nextstrain build script and dependencies are deposited on GitHub and can be accessed via https://github.com/NPPO-NL/nextstrain-Sendo.

Declarations

Conflict of interest The authors declare there are no conflicts of interest.

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