Sequence diversity and potential recombination events in the coat protein gene of Apple stem pitting virus

Beata Komorowska[1], Paweł Siedlecki[2], Szymon Kaczanowski[3], Beata Hasiów-Jaroszewska[4] and Tadeusz Malinowski[1]

[1]Department of Plant Protection, Research Institute of Horticulture, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

[2] Department of Bioinformatics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland

[3]Department of Plant Molecular Biology, Institute of Experimental Plant Biology, University of Warsaw, Miecznikowa 1, 02-096 Warsaw, Poland

[4]Department of Virology and Bacteriology, Institute of Plant Protection-National Research Institute, Węgorka 20, 60-318 Poznań, Poland

SUMMARY

The variability of the *Apple stem pitting virus* (ASPV) coat protein (CP) gene was investigated. The CP gene of ten virus isolates from apple and pear trees was sequenced. Comparison of all sequenced virus isolates revealed high diversity of the CP gene (70.7-93.5 % at the nucleotide level and 77.8-98.7 % at the amino acid level). Additionally, one or two deletions in the N-terminal part of the coat protein gene of the studied virus isolates were identified. The ratio of nonsynonymous to synonymous polymorphic sites indicated that purifying selection has acted to eliminate deleterious mutations in coding sites. Moreover, the evidences for recombination in analyzed sequences were provided. It is likely that recombination, along with selection, enhances the speed of elimination of deleterious mutations in ASPV, following the mutational deterministic hypothesis of Kondrashov.

***Key words*:** ASPV, sequence diversity, selection, recombination

Apple stem pittining virus (ASPV), a member of *Foveavirus* genus in the family *Betaflexiviridae* (Martelli et al., 2007), is one of the most common viruses of apple and pear trees worldwide. ASPV causes xylem pits in the stem of *Malus pumila* Virginia Crab as well as epinasty and decline of *Malus domestica* Spy 227, but remains symptomless in apple cultivars (Nemeth, 1986). This virus is also identified as a causal agent of pear vein

yellows disease (Schwarz and Jelkmann, 1998). There is no known insect vector for this virus (Martelli and Jelkmann, 1998). The ASPV is a filamentous virus with the positive-sense, single-stranded RNA forming 15 nm wide and 800 nm long particles (Koganezawa and Yanase, 1990). The previous results of the genetic diversity of ASPV isolates showed that nucleic acid identity among coat protein (CP) gene sequences was found to be between 81.7 %-99.3 % (Schwarz and Jelkmann, 1998) and 64.8 %-89.6 % (Yoshikawa et al., 2000). Additionally, one or two deletions in the N-terminal part of the viral CP gene were reported (Schwarz and Jelkmann, 1998; Yoshikawa et al., 2000). Although the genetic diversity of ASPV isolates has been studied, the knowledge of the evolutionary history is still lacking. Viral coat proteins are presumed to have evolved more rapidly than proteins involved in replication and expression of virus genomes (Callaway et al., 2001). For this reason, the coat protein genes have been used in the phylogenetic comparisons of different viruses (Koonin and Gorbalenya, 1989; Zimmern, 1988) as well as in this work.

The recombination events could play an important role in deleterious mutation clearance. Such a function of recombination was firstly suggested by Muller (1964) who described a phenomenon termed Muller's ratchet. Muller proposed that in a finite population the best haplotypes might be lost by chance of through recurrent deleterious mutations and without recombination they could not be recreated (Iles et al., 2003). Also according to the modern mutational deterministic hypothesis of Kondrashov (Kondrashov, 1988), recombination is used for deleterious mutation clearance. It should be noted here that some evolutionary biologists use the term "sex" in case of viruses, which is synonymous to genetic exchange. See for example work done by Turner group [Orig Life Evol Biosph. 2003 33:95-108 Turner PE] which studied experimental evolution of sex using viruses as a model. Recombination in natural populations has been reported for several plant viruses (Glasa et al., 2004; Ohshima et al., 2002; Tomitaka and Ohshima, 2006). To our knowledge there is no data on potential recombination between ASPV isolates.

Sequence analysis of the ASPV CP gene has been conducted and the ratio of synonymous and nonsynonymous polymorphisms was calculated. This work also addresses the question what is the pattern of selection shaping the observed diversity of ASPV with a coat protein gene as a model. Additionally, a computational analysis of ASPV sequences to investigate the extent of recombination events as well as to reconstruct the most likely evolutionary history of the recombinants in the population of ASPV genomes has been described.

The virus isolates originated from apple and pear cultivar collection maintained at the Research Institute of Pomology and Floriculture, and from commercial orchards in Poland (Table 1). Isolate N1 was found in the indicator plant, but the original source of the virus was uncertain. Total nucleic acids were isolated from the leaves using the silica capture (SC) method described originally by Boom et al. (1990) and adapted to the diagnosis of plant viruses by Malinowski (1997). Amplification was performed using SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad CA, USA and the primers ASPF2CP2- ASPR3CP (Komorowska et al., 1999). RT-PCR with primer pair ASPB2-ASPB3 yielded the cDNA fragment encompassing viral CP gene of all studied virus isolates. Besides amplification of a 1443 bp product as calculated from the previously sequenced PA66 and PSA-H isolates (Jelkmann, 1994), bands of lower molecular weight were observed with other virus sources. The amplified RT-PCR products were ligated into the pCR 2.1-TOPO vector and used for transformation of TOP10 One-Shot *E. coli* competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad CA, USA). The cloned PCR fragments were sequenced either with universal primers M13 forward and M13 reverse or virus specific primers C8849 (MacKenzie et al., 1997) and ASPV9019 (Schwarz and Jelkmann, 1998) targeting the regions inside the CP gene. Sequencing was performed using an automated sequencer ABI PRISM 310 in the Institute of Biochemistry and Biophysics in Warsaw, Poland. All the sequences were read at least twice. Sequence data were analyzed using Lasergene v. 7.1 software package (DNASTAR, USA). Four samples from apple (MT32, MT24, J335, ST181) and one from an unknown origin (N1) showed an 1389 bp product. Two samples from pear (ST54, GNKIII/45) revealed the cDNA fragments of 1389 bp and 1386 bp respectively and the other two amplicons from pear (ST113, ST132) were 1323 bp long. PCR product of 1320 bp was amplified from pear GNKVII/34. The multiple alignments, including the previously reported sequences of isolates PA66 and PSA-H (Jelkmann, 1994), demonstrated that the observed differences in size of the RT-PCR products were due to deletions in the N-terminal part of the coat protein gene (Table 1). These results are in accordance with the outcomes obtained for ASPV isolates in other centers (Schwarz and Jelkmann, 1998; Yoshikawa et al., 2000). Moreover, the deletion starting from nt 8233 (Table 1) was found only in the CP gene of virus pear isolates (ST113, ST132, GNKVII/34). Similar results have been shown previously for isolate I7 described by Schwarz and Jelkmann, 1998. There is also another ASPV pear isolate in GenBank (accession no. AY572458) with two deletions in CP gene. Thus, it seems that the second

deletion might be unique for pear isolates. On the other hand the number of the characterized ASPV isolates (25) is too small to formulate the general rule. Although plant viruses with RNA genomes have a high potential for genetic variations (Garcia-Arenal et al., 2003) there are only a few examples of large natural deletions in the CP gene (Seifers et al., 2005; Szathmary et al., 2009).

Following the reading of CP gene sequences for ten ASPV isolates, the comparison among them and with other virus sequences from GenBank (accession nos. D21828, D21829, AB045371, EU708018, EU314950, DQ003336, AY572458) was carried out. Multiple sequence alignments were performed using the online service CLUSTALW (Thompson et al., 1994). The multiple alignments revealed low identity between ten studied virus sequences ranging from 70.7 % to 93.5 % at nucleotide level and from 77.8 % to 98.7 % at amino acid level. Similar ranges of values were obtained when comparisons were made with corresponding sequences available in GenBank. The percentage of nucleic acid identities among the sequences of the CP gene of all ASPV isolates was found to be between 71 % and 99.8 %. Comparison of amino acid sequences showed higher identities 78.2-99.5 %. Similar high levels of CP gene diversity are described for other members of the family *Betaflexiviridae* (Alabi et al., 2010; Teycheney et al., 2005). The lack of a biological vector for transmission of ASPV probably makes the CP more tolerant to sequence variability than the capsid proteins of vector borne plant viruses subjected to greater purifying selection on amino acid changes (Garcia-Arenal et al., 2001; Rubio et al., 2001; Vives et al., 2002).

All phylogenetic analyses were conducted in Datamonkey (Kosakovsky Pond and Frost, 2005a) which is a front-end toHyPhy (Kosakovsky Pond et al., 2005). HKY85 was chosen as the nucleotide substitution bias model, as our data set was rather small. This model was used in all phylogenetic studies. The recognized deletions were discarded from the CP sequences in the following analyses.

It is well known that recombination can affect obtained values for omega and phylogenetic reconstruction (Silva and Edwards, 2009). Therefore, the ASPV CP genes were initially analyzed for evidence of recombination events. The GARD (genetic algorithm recombination detection) and the SBP (single breakpoint recombination) methodologies were used to determine if recombination events are present in the studied sequences (Kosakovsky Pond and Frost 2005b; Kosakovsky Pond et al., 2006). Both methods differently identify non-recombinant fragments in the alignment and reconstruct a phylogenetic tree for each of them. Each of the subsequent experiments was repeated

both with GARD and SBP tree sets. The quality of evolutionary trees was measured using minimum AIC values. If AIC for non-recombinant tree is smaller than AIC for a tree with recombination event the delta AIC should be high. The SBP algorithm found evidence of recombination at position 604 (with a high delta AIC of 854). The GARD algorithm was able to find two possible recombination sites within ASPV capsid sequences in positions 618 (p=0.0008; high confidence, small delta AIC) and 760 (p=0.048; medium confidence and small delta AIC) (data not shown). The results from both experiments (the low p-value and substantial improvement relative to the model with one fewer breakpoint - delta AIC) suggest strongly that there was a possible recombination event around position 604-618. Taken these findings, all of the further conducted analysis used the SBP and GARD Inferred Trees as models to overcome the noise introduced by recombination. Recombination events occur frequently in viruses, both from plants and animals (Lai, 1992). It is well known that sex and recombination lead to the increase of genetic diversity in populations (Grapputo et al., 2005), and that mutation and recombination events are particularly beneficial in the case of genes which are under diversifying selection, e.g. animal virus genes. It is harder to understand what the function is of recombination and sex, when purifying selection dominates. In fact, this is a common pattern in the case of cellular organisms (Castillo-Davis et al., 2004). An explanation for the function of sex and recombination in this case was suggested by Kondrashov (Kondrashov, 1988). His mutational deterministic hypothesis was primarily proposed for evolution of sexual reproduction. According to this hypothesis, mutations are mainly deleterious, which cause mutational loads and create isolates containing many slightly deleterious mutations. On the other hand, recombination creates genotypes with different levels of deleterious mutations. Selection forces remove genotypes with a higher number of deleterious mutations. The Kondrashov hypothesis may explain recombination events discovered in our study. The CP genes from various ASPV strains are under strong purifying selection, which means that mutations are mainly deleterious and are removed by natural selection. There is also at least one recombination event in the studied gene. It might be possible that the function of recombination in such cases is enhancing the speed of removing deleterious mutations by selection.

The following methodologies: SLAC (single-likelihood ancestor counting), FEL (fixed effects likelihood) and REL (random effects likelihood) were used to determine sites under positive selection (Kosakovsky Pond and Frost 2005). All these methods estimate the number and ratio of nonsynonymous and synonymous substitutions for each

site (codon). A consensus scoring approach was applied to determine the sites most probably experiencing positive selection. All three methods found codon 20 to be under positive selection. Additionally codon 111 was identified by SLAC, and codon 340 by FEL - although the dN>dS difference in this case was not significant (Table. 2). The obtained results suggest that there is one position (codon 20) which is under positive selection. Both SLAC and FEL methods used default p-value of 0.1, and REL used Bayes Factor of 40.

The PARRIS (a partitioning approach for robust inference of selection) methodology, described by Scheffler et al., (2006), was used to test if the detected sites (codon 20, 111 and 340) will be robust enough to pass through a maximum likelihood test, designed specifically to avoid misleading recombination events. PARRIS allows synonymous substitution rates to vary across sites as well as tree topologies and branch lengths to change across detected recombination breakpoints. The PARRIS methodology did not detect any positive selection signals at $p<0.1$ in the compared ASPV CPs.

The codon-based genetic algorithm GABranch method was used to evaluate if the studied isolates evolve due to positive selection. This method can partition automatically all branches of the phylogeny describing non-recombinant data into groups according to dN/dS. The most probable recombination site according to SBP and GARD consensus located between bases 604-618 was used. Therefore, base 612 was chosen as the cutting alignment site to avoid a frameshift. The two resulting alignments were subjected to GABranch algorithm and the results were compared using omega values. As it is shown on Fig. 1, all branches evolve with omega <1, suggesting that purifying selection occurred during the whole analyzed history of ASPV CP. This analysis has indicated that the majority of mutations occurring in analyzed sequences are deleterious. Similar high levels of nucleotide sequence variability as well as low values of d_N/d_S have been reported in different genomic regions for other members of the family *Betaflexiviridae* (Chare and Holmes, 2006; Teycheney et al., 2005), indicating that the attribute is common to the members of this family.

In this paper we showed that ratio of synonymous and nonsynonymous mutations suggests that purifying selection dominates in the case of ASPV. Four different approaches detected strong signal of purifying selection for the majority of codons. Similar results were described for coat protein genes of *Grapevine rupestris stem pitting-associated virus* (Alabi et al., 2010) and *Sweet potato mild mottle virus* (SPMMV) (Tugume et al., 2010). In contrast, animal viruses experience strong diversifying selection

(Drummond et al., 2003; Yang and Bielawski, 2000) which is caused predominantly by pressure from the immune system of the host. Plant viruses are not subjected to such pressure thus purifying selection is the major force shaping their evolution, the coat protein of the ASPV is no exception.

Although purifying selection dominates in the evolution of ASPV capsid protein, three sites having a diversifying selection signal (codon 20, 111 and 340) were detected. This signal may be significant especially in the case of codon number 20, as positive selection was detected using three (SLAC, REL, FEL) out of four methods. Such phenomenon was also observed for SPMMV where 13 amino acids in the P1 proteinase were under positive selection, whereas purifying selection was implicated for the rest of the sites (Tugume et al., 2010). It is not surprising that various codons are under different selection forces. By analogy to animal viruses, it is likely that codon 20 is involved in reaction with host factors. Host resistance may lead to diversifying selection in this position. It is also possible that the amino acid coded by codon 20 is on the surface of capsid protein. Analysis of capsid three-dimensional structure may confirm this hypothesis. Unfortunately there are no proper templates which could be applied for homology modeling of this protein to date. Mutational studies should be conducted to gain more insight about the possible importance of this part of the capsid protein.

References

Alabi, O.J., Martin, R.R., Naidu, R.A., 2010. Sequence diversity, population genetics and potential recombination events in grapevine rupestris stem pitting – associated virus in Pacific North - West vineyards. J. Gen. Virol. 91, 265-276.

Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-Van Dillen, P.M.E., van der Nordaa, J., 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28, 495-503.

Callaway, A., Giesman-Cookmeyer, D., Gillock, E.T., Sit., T.L., Lommel, S.A., 2001. The multifunctional capsid proteins of plant RNA viruses. Annu. Rev. Phytopathol. 39, 419-460.

Castillo-Davis, C.I., Kondrashov, F.A., Hartl, D.L., Kulathinal, R.J., 2004. The functional genomic distribution of protein divergence in two animal phyla: coevolution, genomic conflict, and constraint. Genome Res. 14, 802–811.

Chare, E.R., Holmes, E.C., 2006. A phylogenetic survey of recombination frequency in plant RNA viruses. Arch. Virol. 151, 933-946.

238 Drummond, A., Pybus, O.G., Rambaut, A., 2003. Inference of viral evolutionary
239rates from molecular sequences. Adv. Parasitol. 54**,** 331-358.
240 Garcia-Arenal, F., Fraile, M., Malpica, J. M., 2001. Variability an genetic structure
241of plant virus populations. Ann. Rev. Phytopathol. 39, 157-186.
242 Garcia-Arenal, F., Fraile, M., Malpica, J. M., 2003. Variations and evolution of
243plant virus populations. Int. Microbiol. 6, 225-232.
244 Glasa, M., Palkovics, L., Komínek, P., Labonne, G., Pittnerová, S., Kúdela, O.,
245Candresse, T., Šubr, Z., 2004. Geographically and temporally distant natural recombinant
246isolates of *Plum pox virus* (PPV) are genetically very similar and form a unique PPV
247subgroup. J. Gen. Virol. 85, 2671-2681.
248 Grapputo, A., Kumpulainen, T., Mappes, J., Parri, S., 2005. Genetic diversity in
249populations of asexual and sexual bag worm moths (Lepidoptera: Psychidae). BMC
250Ecology 5: 5 doi:10.1186/1472-6785-5-5.
251 Iles, M., Walters, K., Cannings, C., 2003. Recombination can evolve in large finite
252populations given selection on sufficient loci. Genetics 165, 2249-2258.
253 Jelkmann, W., 1994. Nucleotide sequence of apple stem pitting virus and of the
254coat protein gene of a similar virus from pear associated with vein yellows disease and
255relationship with potex- and carlaviruses. J. Gen. Virol. 75, 1535-1542.
256 Koganezawa, H., Yanase, H., 1990. A new type of elongated virus isolated from
257apple trees containing the stem pitting agent. Plant Dis. 74, 610-614.
258 Komorowska, B., Malinowski, T., Zawadzka B., 1999. Amplification of the coat
259protein gene of *Apple stem pitting virus* (ASPV). Phytopath. Pol. 17, 49-59.
260 Kondrashov, A.S., 1988. Deleterious mutations and the evolution of sexual
261reproduction. Nature 336, 435-440.
262 Koonin, E.V., Gorbalenya, A.E., 1989. Evolution of RNA genomes does the high
263mutation rate necessitate high rate of evolution of virus protein? J. Mol. Evol. 28, 524-
264527.
265 Kosakovsky Pond, S.L., Frost, S.D.W., 2005a. Datamonkey: rapid detection of
266selective pressure on individual sites of codon alignments. Bioinformatics 21, 2531-2533.
267 Kosakovsky Pond, S.L., Frost S.D.W., 2005b. Not so different after all: A
268comparison of methods for detecting amino acid sites under selection. Mol. Biol. Evol. 22,
2691208-1222.
270 Kosakovsky Pond, S.L., Frost, S.D.W., Muse, S.V., 2005. HyPhy: hypothesis
271testing using phylogenies. Bioinformatics 21, 676-679.

272 Kosakovsky Pond, S.L., Posada, D., Gravenor, M.B., Woelk, Ch.H., Frost,
273S.D.W., 2006. Automated phylogenetic detection of recombination using a genetic
274algorithm. Mol. Biol. Evol. 23, 1891-1901.
275 Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev.
27656, 61-79.
277 MacKenzie, D.J., McLean, M.A., Mukerji, S., Green, M., 1997. Improved RNA
278extraction from woody plants for the detection of viral pathogens by reverse transcription-
279polymerase chain reaction. Plant Dis. 81, 222-226.
280 Malinowski, T., 1997. Silicacapture - reverse transcription - polymerase chain
281reaction (SC-RT-PCR): application for the detection of several plant viruses, in Dehne,
282H.W., Adam, G., Diekmann, M., Frahm, J., MaulerMachnik, A., VanHalteren, P. (Eds.),
283Diagnosis and Identification of Plant Pathogens, Proceedings of 4th International EFPP
284Symposium Bonn, 9-12 September 1996. Kluwer Academic Publishers, Budapest, pp.
285445-448.
286 Martelli, G.P., Jelkmann, W., 1998. *Foveavirus*, a new plant virus genus. Arch.
287Virol. 143, 1245-1248.
288 Martelli, G.P., Adams, M.J., Kreuze, J.F., Dolja, V.V., 2007. Family *Flexiviridae*:
289a case study in virion and genome plasticity. Annu. Rev. Phytopathol. 45, 73-100.
290 Muller, H.J., 1964. The relation of recombination to mutational advance. Mutat.
291Res. 1, 2-9.
292 Nemeth, M., 1986. Apple stem pitting virus, in: Memeth, M. (Ed), Virus,
293mycoplasma and rickettsia diseases of fruit trees. Akademiai Kiado, Budapest, pp.216-
294219.
295 Ohshima, K., Yamaguchi Y., Hirota, R., Hamamoto, T., Zhongyang, T., Sano, T.,
296Azuhata, F., Walsh, J., Fletcher, Chen, J., Gera, A., Gibbs, A., 2002. Molecular evolution
297of *Turnip mosaic virus*: evidence of host adaptation, genetic recombination and
298geographical spread. J. Gen. Virol. 83, 1511-1521.
299 Rubio, L., Abou-Jawdah, Y., Lin, H., Falk, B., 2001. Geographically distant
300isolates of the crinivirus Cucurbit yellows stunting disorder virus show very low genetic
301diversity in the coat protein gene. J. Gen. Virol. 82, 929-933.
302 Scheffler, K, Martin, D.P., Seoighe, C., 2006. Robust inference of positive
303selection from recombining coding sequences. Bioinformatics 22, 2493-2499.

304 Schwarz, K., Jelkmann, W., 1998. Detection and characterization of apple stem
305pitting virus sources from apple and pear by PCR and partial sequence analysis. Acta
306Hort. 472, 75-85.
307 Seifers, D.L., Haber, S., Ens, W., She, Y.M., Standing, K.G., Salomon, R., 2005.
308Characterization of a distinct Johnsongrass mosaic virus strain isolated from sorghum in
309Nigeria. Arch. Virol. 150, 557-576.
310 Silva, M.C., Edwards, S.V., 2009. Structure and Evolution of a New Avian MHC Class II
311B Gene in a Sub-Antarctic Seabird, the Thin-Billed Prion (Procellariiformes: Pachyptila
312belcheri). J. Mol. Evol. 68, 279-291.
313 Szathmary, E., Nadudvari, J.N., Szabo, L., Tobias, I., Balazs, E., Palkovics, L., 2009.
314Characterization of a natural Plum pox virus isolate bearing a truncated coat protein. Arch. Virol.
315154, 141-145.
316 Teycheney, P.Y., Laboureau, N., Iskre-Caruana, M.L., Candresse, T., 2005. High
317genetic variability and evidence for plant to plant transfer of *Banana mild mosaic virus*. J.
318Gen. Virol. 86, 3179-3187.
319 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTALW. Improving the
320sensitivity of progressive multiple sequence alignment through sequence weighting,
321positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-
3224680.
323 Tomitaka, Y., Ohshima, K., 2006. A phylogeographical study of the Turnip
324mosaic virus population in East Asia reveal an ‘emergent’ lineage in Japan. Mol. Ecol. 15,
3254437-4457.
326 Tugume, A.K., Settumba, M.B., Kalkkinen, N., Valkonen, J.P.T., 2010.
327Recombination and selection pressure in the ipomovirus sweet potato mild mottle virus)
328(*Potyviridae*) in wild species and cultivated sweet potato in the centre of evolution in East
329Africa. J. Gen. Virol. 91, 1092-1108.
330 Vives, M.C., Rubio, L., Galipienso, L., Navarro, L., Moreno, P., Guerri J., 2002.
331Low genetic variation between isolates of *Citrus leaf blotch virus* from different host
332species and of different geographical origins. J. Gen. Virol. 83, 2587-2591.
333 Yang, Z., Bielawski, J.P., 2000. Statistical methods for detecting molecular
334adaptation. Trends in Ecology & Evolution 15, 496-503.
335 Yoshikawa, N., Matsuda, H., Oda, Y., Isogai, M., Takahashi, T., Ito, T., Yoshida
336K., 2000. Genom heterogeneity of *Apple stem pitting virus* in apple trees. Acta Hort. 550,
337285-290.

338 Zimmern, D., 1988. Evolution of RNA viruses, in: Holland, J.J., Domingo, E.R.,
339 Ahlquist, P. (Eds.), RNA genetics. CRC, Boca Raton, pp. 211-240.
340
341 **Tables:**

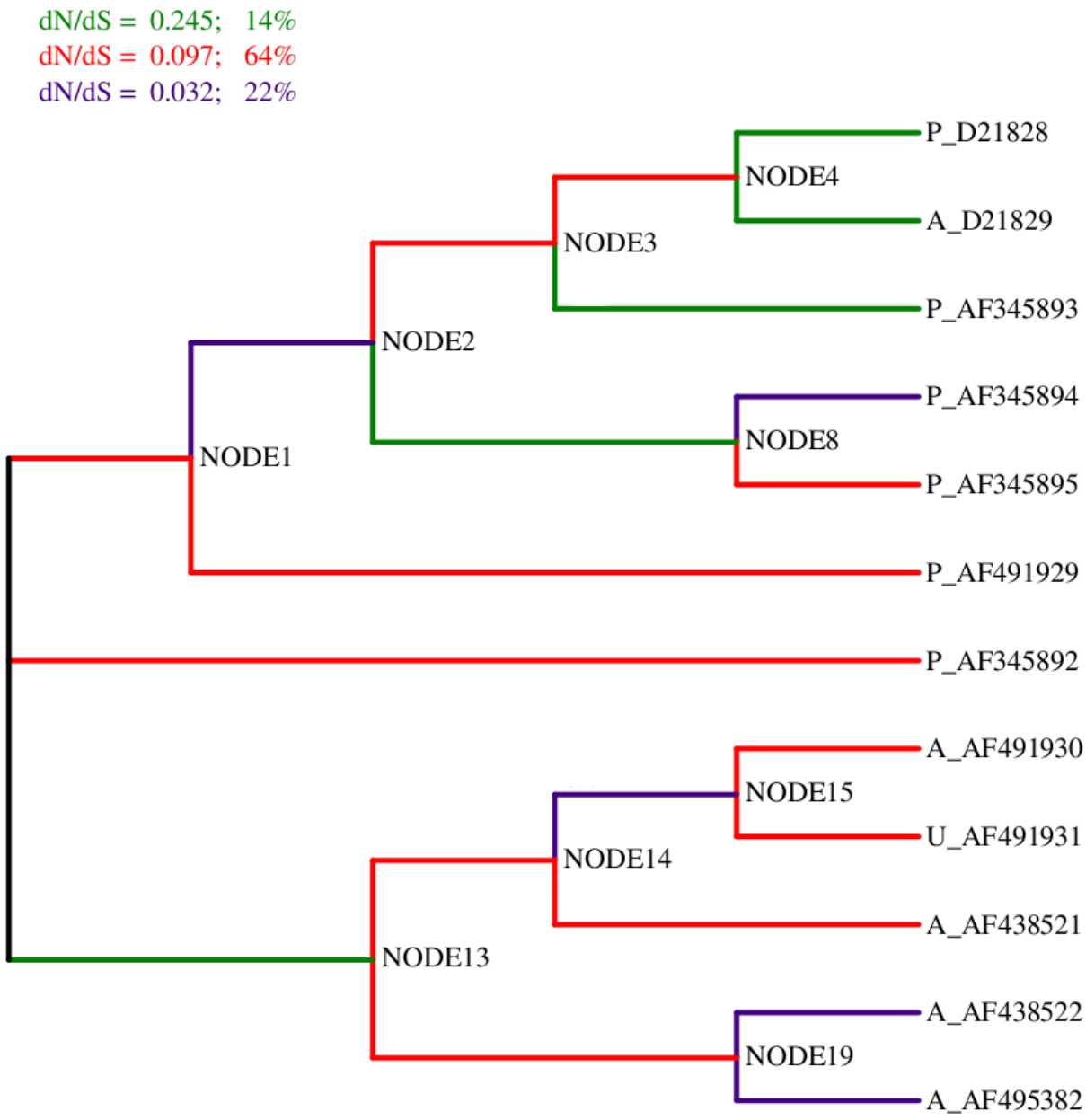
Isolate	Accession number	Original	CP gene length [nt]	Position of deletion(s)[b]
PSA-H[a]	D21828	pear	1245	-
ST54	AF345892	pear	1191	8006-8059
ST113	AF345894	pear	1125	8006-8059 8233-8298
ST132	AF345894	pear	1125	8006-8059 8233-8298
GNKIII/45	AF491929	pear	1185	8487-8493
GNKVII/34	AF345893	pear	1128	8006-8059 8233-8295
PA66[a]	D21829	apple	1245	-
MT32	AF438521	apple	1191	8006-8059
J335	AF491930	apple	1191	8006-8059
MT24	AF438522	apple	1191	8006-8059
ST181	AF495382	apple	1191	8006-8059
N1	AF491931	unknown	1191	8006-8059

342
343
344
345 **Table 1.** Characteristics of a coat protein gene of the studied ASPV isolates.
346 [a]Reference strains kindly provided by W. Jelkmann
347 [b]Corresponding to the sequence of ASPV PA66 (GenBank accession no. D21829)
348
349
350
351
352

Codon	SLAC dN-dS	SLAC p-value	FEL dN-dS	FEL p-value	REL dN-dS	REL Bayes Factor	Consensus
20	1.705	0.135	0.826	0.027	0.108	40.416	P, P+, P+
111	1.222	0.056	-0.651	0.321	-0.533	0.125	P+, N, N
340	-3.778	1.000	0.104	0.354	-0.182	41.056	N, P, N-

Table 2

The results obtained from three methods: SLAC, FEL and REL used to determine possible positive selection in ASPV CP. For each method, two values are present: omega and p-value; with the exception of REL methodology, which uses Bayes Factor instead of p-value. The consensus column, shows which of the methods indicate a certain codon is under positive selection; “P”: positive selection, "P+": statistical significance of such prediction, “N”: negative selection, "N-":statistical significance.



382

383**Figure caption:**

384

385**Figure 1.** GABranch analysis showing a Neighbor-Joining tree with levels of omega 386coded by colors. Green branches represent the highest dN/dS values, red and purple 387colored branches indicate medium to low values, respectively. All the values in this 388GABranch analysis show omega below 1, suggesting strong purifying selection forces 389acted during the analyzed ASPV CP history. The tree clearly divides apple and pear virus

390isolates into two separate branches. It might also suggest that isolate of unknown origin
391(AF491931) was originally isolated from apple. Also, position of isolates AF345892 and
392D21829 in the tree could imply recombination events.

393

394