Journal of General Virology (2004), **85**, 3399–3403 DOI 10.1099/vir.0.80182-0

Short Communication

Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs. II. Characterization of a new species in the genus *Partitivirus*

Robert H. A. Coutts,[1] Laura Covelli,[2,3] Francesco Di Serio,[4] Ahmet Citir,[5] Serap Açıkgöz,[6] Carmen Hernández,[2] Antonio Ragozzino[3] and Ricardo Flores[2]

Correspondence
Ricardo Flores
rflores@ibmcp.upv.es

[1]Department of Biological Sciences, Imperial College London, Imperial College Road, London SW7 2AZ, UK

[2]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, Avenida de los Naranjos s/n, 46022 Valencia, Spain

[3]Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli, 80055 Portici, Italy

[4]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Sezione di Bari, 70126 Bari, Italy

[5]Tekirdag Ziraat Fakültesi, Trakya Universitesi, 59030 Tekirdag, Turkey

[6]Adnan Menderes University, Agricultural Faculty, Plant Pathology Department, 09100 Aydin, Turkey

Received 8 April 2004
Accepted 19 July 2004

Two dsRNAs from cherry trees affected with cherry chlorotic rusty spot (CCRS) in Italy and Amasya cherry disease (ACD) in Turkey were sequenced and found to be essentially identical. The larger dsRNA 1 (2021 or 2006 bp, respectively) potentially encoded a protein of 621 aa containing the conserved motifs of the RNA-dependent RNA polymerases (RdRp) of dsRNA mycoviruses, having highest similarity with those in the genus *Partitivirus*. The smaller dsRNA 2 (1841 or 1839 bp, respectively) had properties consistent with the second genomic component of a partitivirus and potentially encoded the coat protein (CP) of 504 aa. Phylogenetic analysis based on the RdRp and CP was coincidental and indicated that species in the genus *Partitivirus* could be separated into two subgroups. Because species of this genus only infect fungi, these observations suggest a fungal aetiology for CCRS and ACD, further substantiating a previous proposal (see accompanying paper by Covelli *et al.*, 2004, in this issue).

In the accompanying paper (Covelli *et al.*, 2004), we illustrated that two cherry diseases, cherry chlorotic rusty spot (CCRS) from Italy and Amasya cherry disease (ACD) from Turkey are very similar diseases probably caused by a fungus (Alioto *et al.*, 2003), which, in turn, is associated with multiple species of double-stranded RNAs (dsRNAs), presumably of viral origin (Covelli *et al.*, 2004; Di Serio *et al.*, 1996, 1998). In confirmation of the mycoviral nature of these dsRNAs, we reported that four of them comprised the genome of a new species in the genus *Chrysovirus* (Covelli *et al.*, 2004; Ghabrial & Castón, 2004) and here we have shown that two other dsRNAs comprise the genome of a new species in the genus *Partitivirus*.

Members of the family *Partitiviridae* (Ghabrial *et al.*, 2004), which together with the *Chrysoviridae*, *Hypoviridae* and *Totiviridae* constitute the four fungal dsRNA virus families (Ghabrial, 2001; Ghabrial & Castón, 2004), are isometric, non-enveloped viruses, 30–40 nm in diameter, with a genome composed of two linear dsRNAs of approximately 1400–3000 bp (Ghabrial *et al.*, 2004). The smaller dsRNA encodes the coat protein (CP) and the larger the virion-associated RNA-dependent RNA polymerase (RdRp) (Ghabrial *et al.*, 2004). Additional satellite or defective

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AJ781168 and AJ781167 for ACD-associated dsRNAs 1 and 2 and AJ781401 and AJ781402 for CCRS-associated dsRNAs 1 and 2.

RNAs may also be present. The family *Partitiviridae* has been divided into four genera, *Partitivirus* and *Chrysovirus* for viruses that infect fungi, and *Alphacryptovirus* and *Betacryptovirus* for viruses that infect plants (Ghabrial, 2001). Recently, the genus *Chrysovirus* was removed from the family *Partitiviridae* and placed into the new family *Chrysoviridae* (Ghabrial & Castón, 2004), which, in addition to *Penicillium chrysogenum virus* (PcV) (Jiang & Ghabrial, 2004), the type species, and *Helminthosporium victoriae 145S virus* (Hv145SV) (Ghabrial & Castón, 2004), now includes a tentative new species present in dsRNA extracts produced from ACD- and CCRS-affected cherry trees (Covelli *et al.*, 2004). Most of these viruses cause latent infections and may have originated from totiviruses (Ghabrial, 1998). We previously suggested that two of the 10–12 dsRNAs (of approx. 1800 and 2000 bp) associated with both of the above diseases probably comprised the genome of a partitivirus (see accompanying paper by Covelli *et al.*, 2004, in this issue) and here we report its molecular characterization.

The dsRNAs were isolated from infected sweet cherry leaves collected in Turkey and Italy exhibiting typical symptoms of ACD and CCRS, respectively, and after separation by electrophoresis on polyacrylamide or agarose gels they were either collectively or individually used for reverse transcription, PCR amplification, cloning and sequencing as described previously (Covelli *et al.*, 2004). cDNA clones of the presumed ACD-associated partitivirus dsRNAs 1 and 2 were obtained by random priming of methyl mercuric hydroxide-denatured dsRNA, with further DNA manipulations being performed as previously described (Sambrook *et al.*, 1989). For the synthesis of additional cDNAs covering their complete sequence, purified dsRNAs 1 and 2 of the presumed ACD-associated partitivirus were denatured with methyl mercuric hydroxide and subjected to a single-primer, genome-walking RT-PCR protocol developed previously to sequence plant virus RNAs (Livieratos *et al.*, 1999). An RNA ligase-mediated (RLM)-RACE PCR procedure was used to determine the 5′- and 3′-terminal sequences of the dsRNAs (Coutts & Livieratos, 2003). The sequences of the equivalent CCRS-associated dsRNAs were obtained by the single-primer method as described previously (Covelli *et al.*, 2004). Nucleotide and amino acid sequences were manipulated (Deveraux *et al.*, 1984), aligned using CLUSTAL X (Thompson *et al.*, 1997) and drawn using the MEGA 2 program (Kumar *et al.*, 2001).

The complete dsRNA 1 sequences of the presumed ACD- and CCRS-associated partitiviruses were 2006 and 2021 bp, respectively, and each included one ORF potentially encoding a putative protein of 621 aa with a molecular mass of approximately 73 kDa. The two predicted proteins were 98 % identical to one another and contained the conserved motifs characteristic of the RdRps of partitiviruses and dsRNA viruses infecting simple eukaryotes (Bruenn, 1993) (Fig. 1a). A phylogenetic reconstruction using the fragments containing these conserved motifs from some representative toti-, chryso- and partitiviruses provided corroboration that the presumed ACD- and CCRS-associated partitiviruses clustered together with members of this group (Covelli *et al.*, 2004). To delimit more precisely the relationships within the genus *Partitivirus*, a phylogenetic tree was constructed based on multiple alignment of the fragments containing the RdRp conserved motifs from all species of this genus deposited in databases using *Beet cryptic virus* (BCV-3) as an outgroup. The tree showed that ACD- and CCRS-associated partitiviruses formed a subgroup with the *Helicobasidium mompa virus* strain V70 (HmV-V70) (Osaki *et al.*, 2002), the *Heterobasidion annosum virus* P-type (HaV-Pt) (Ihrmark, 2001), the *Atkinsonella hypoxylon virus* isolate 2H (AhV-2H) (Oh & Hillman, 1995), the *Fusarium poae virus 1* (FpV-1) (Compel *et al.*, 1999) and the *Rhizoctonia solani virus 717* (RhsV-717) (Strauss *et al.*, 2000), with the remaining five members of the genus forming a second subgroup (Fig. 1b).

The complete dsRNA 2 sequences of the ACD- and CCRS-associated partitiviruses were 1839 and 1841 bp, respectively, and each included one ORF potentially encoding a putative CP of 504 aa with a molecular mass of approximately 55·5 kDa. The two predicted proteins were 94 % identical to one another, confirming the very close relationship between the two partitiviruses, which can be considered as different isolates of the same virus. A comparison with the dsRNA 2 (and the putative encoded proteins) of the other partitiviruses deposited in databases showed that ACD- and CCRS-associated partitiviruses clustered in a subgroup characterized by having larger dsRNAs and predicted proteins also larger and with lower isoelectric points (Table 1). Multiple alignments of the corresponding amino acid sequences also supported the existence of two subgroups, one with extensive sequence identity/similarity in several regions of the protein (Fig. 2a), and a second subgroup with considerably lower sequence conservation (Fig. 2b). Within this second subgroup, the putative CP of the ACD- and CCRS-associated partitiviruses, which shared an identity/similarity of 32/41 % with that of AhV-2H, the type species of the genus *Partitivirus* (Ghabrial *et al.*, 2004), appeared as the most closely related to the proteins of the other subgroup.

For both ACD- and CCRS-associated partitiviruses, the sizes of the 5′-untranslated regions (5′-UTRs) of dsRNAs 1 and 2 were 89 and 120 nt, respectively, and contained the CAA repeats reported previously in another partitivirus (Strauss *et al.*, 2000). The lengths of the 3′-UTRs of the same molecules were 50 and 203 nt for the ACD isolate and 66 and 206 nt for the CCRS isolate. The 5′-terminal 12 nt were identical in the two dsRNAs of both partitiviruses but the 3′-UTRs were apparently less conserved. Nevertheless, the 3′-UTRs of both partitiviruses contained polyadenylated stretches in dsRNAs 1 and 2 and were A/U rich, a situation similar to that found in the 3′-UTRs of the dsRNAs of other partitiviruses (Oh & Hillman, 1995; Osaki *et al.*, 2002; Strauss *et al.*, 2000).

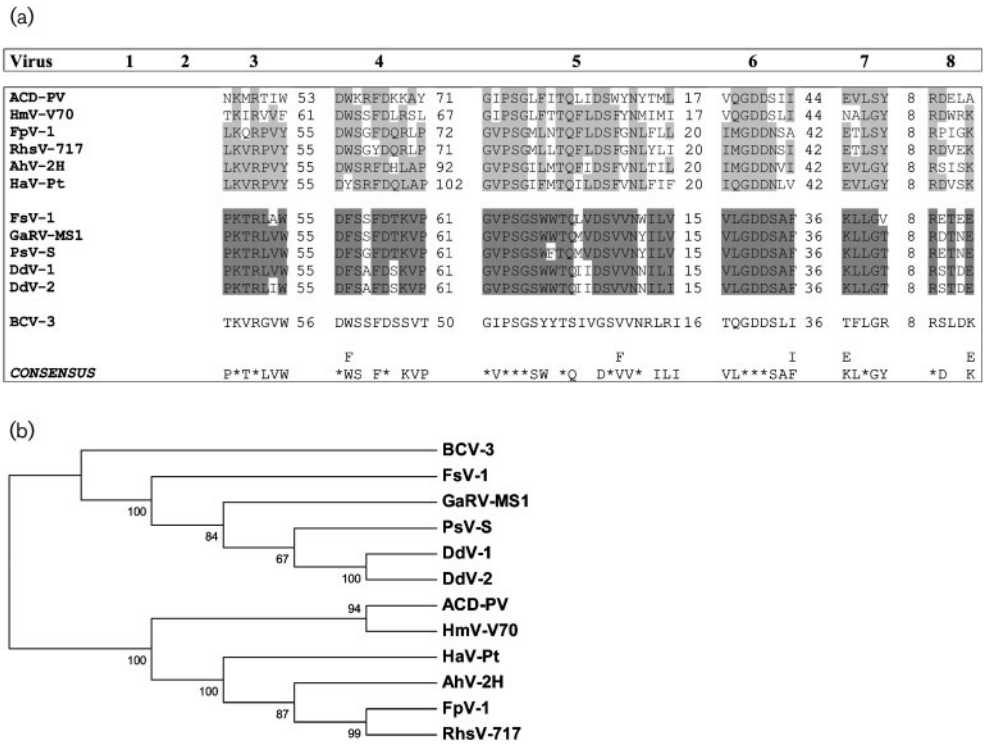

Fig. 1. Relationships between the CCRS- and ACD-associated partitiviruses and the other species in the genus *Partitivirus*. (a) Multiple alignment of a fragment of the putative RdRp encoded by CCRS-and ACD-associated partitivirus dsRNA 1 with the RdRps of the other partitiviruses. GenBank accession numbers were as follows. Upper subgroup: Amasya cherry disease associated partitivirus (ACD-PV; AJ781168), HmV-V70 (AB025903), FpV-1 (AF047013), RhsV-717 (AF133290), AhV-2H (L39125) and HaV-Pt (AF473549). Lower subgroup: *Fusarium solani virus 1* (FsV-1; D55668), *Gremmeniella abietina RNA virus MS1* (GaRV-MS1; AY089993), *Penicillium stoloniferum virus S* (PsV-S; AY156521), *Discula destructiva virus 1* (DdV-1; AF316992) and DdV-2 (AY033436). BCV-3 (S63913), a member of the family *Partitiviridae* infecting plants, was used as an outgroup sequence. Numbers on top refer to the conserved motifs characteristic of the RdRps of partitiviruses and dsRNA viruses of simple eukaryotes (Bruenn, 1993; Ghabrial, 1998) (motifs 1 and 2 were not included because they are poorly defined in partitiviruses). The numbers of residues between the conserved motifs are indicated. In the consensus sequence, asterisks denote identical residues and letters denote residues conserved in at least five of the sequences. Within each subgroup, which are separated by a space, residues conserved in at least 50 % of the sequences are indicated in light grey (upper subgroup) or dark grey (lower subgroup). (b) Phylogenetic tree based on the neighbour-joining method with a 10 000 replicate bootstrap search. Bootstrap values are indicated at the branch points.

Table 1. Physical properties of dsRNA 2 and its potentially encoded proteins for members of the genus *Partitivirus*

Virus*	Size (bp)†	Size of the putative CP (kDa)‡	Isoelectric point	GenBank accession no.
FsV-1	1445	44·2 (413 aa)	8·27	D55669
GaRV-MS1	1586	47·1 (443 aa)	8·04	AY089994
PsV-S	1523	46·8 (434 aa)	7·16	AY156522
DdV-2	1611	47·9 (442 aa)	7·04	AY033437
DdV-1	1585	47·6 (436 aa)	6·73	AF316993
RhsV-717	2206	76·4 (683 aa)	6·38	AF133291
AhV-2H	2135	73·7 (652 aa)	6·07	L39126
FpV-1	2185	70·4 (637 aa)	6·01	AF015924
ACD-PV	1839	55·4 (504 aa)	5·89	AJ781167

*For virus names see legend to Fig. 1 and main text.

†Determined by nucleotide sequencing of cDNAs clones covering the complete length of the dsRNAs.

‡Determined from the amino acid sequence deduced from the cDNAs (the number of amino acid residues is indicated in parentheses).

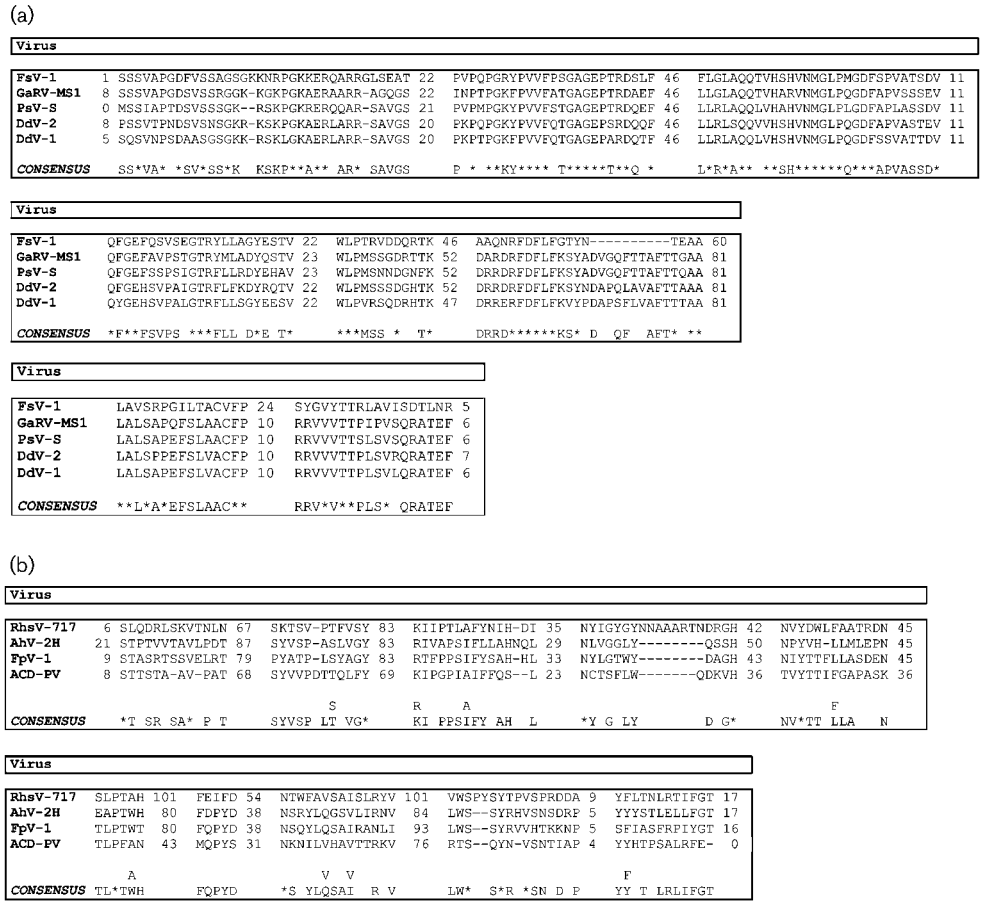

Fig. 2. Relationships between the CCRS- and ACD-associated partitiviruses and the other species in the genus *Partitivirus* inferred from the predicted sequences of their putative CPs. Regions derived from multiple alignments of the available sequences that allowed the distinction of the two subgroups are shown. For virus names and accession numbers, see legend to Fig. 1 and Table 1, respectively. Regions displaying different sequence conservation are presented within each subgroup. Asterisks denote identical residues and letters denote conserved residues in at least three (a) or two (b) of the sequences.

In summary, we have characterized the two dsRNA components of the ACD- and CCRS-associated partitiviruses. The phylogenetic analysis derived from the RdRp potentially encoded by dsRNA 1 of the known partitiviruses was essentially coincidental with the current taxonomy of the genus (Ghabrial *et al.*, 2004) and located the ACD- and CCRS-associated partitiviruses in one specific subgroup. Interestingly, a parallel analysis based on the more limited data available for the putative CP potentially encoded by dsRNA 2 also led to the same subgroups, thus providing additional support for the present classification scheme. Within this scheme, ACD- and CCRS-associated partitiviruses clustered in the same subgroup with a predominance of dsRNA viruses that infect basidiomycetous fungi (HmV-V70, HaV-Pt and RhsV-717). What significance in evolutionary terms this has necessitates the isolation and molecular characterization of further partitiviruses from a variety of fungal species in the future.

Acknowledgements

Work in the laboratories of R. F. and A. R. was partially supported by the Generalidad Valenciana (España) and the Ministero delle Politiche Agricole e Forestali (Italia), respectively. We thank Dr D. Alioto for detailed information about the fungus-like organism found in CCRS and ACD symptomatic leaves. During this work, L. C. was the recipient of pre-doctoral fellowships from the Marie Curie Program (European Union) and from the Università 'Federico II' di Napoli (Italia).

References

Alioto, D., Zaccaria, F., Covelli, L., Ragozzino, A., Di Serio, F. & Milne, R. G. (2003). Light and electron microscope observations on chlorotic rusty spot, a disorder of cherry in Italy. *J Plant Pathol* **85**, 215–218.

Bruenn, J. A. (1993). A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. *Nucleic Acids Res* **21**, 5667–5669.

Compel, P., Papp, I., Bibo, M., Fekete, C. & Hornok, L. (1999). Genetic interrelationships and genome organization of double-stranded RNA elements of *Fusarium poae*. *Virus Genes* **18**, 49–56.

Coutts, R. H. A. & Livieratos, I. C. (2003). A rapid method for sequencing the 5′- and 3′-termini of dsRNA viral templates using RLM-RACE. *J Phytopathol* **151**, 525–527.

Covelli, L., Coutts, R. H. A., Di Serio, F., Citir, A., Açıkgöz, S., Hernández, C., Ragozzino, A. & Flores, R. (2004). Cherry chlorotic rusty spot and Amasya cherry diseases are associated with a complex pattern of mycoviral-like double-stranded RNAs. I. Characterization of a new species in the genus *Chrysovirus*. *J Gen Virol* **85**, 3389–3397.

Deveraux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387–395.

Di Serio, F., Flores, R. & Ragozzino, A. (1996). Cherry chlorotic rusty spot: description of a virus-like disease from cherry and studies on its etiological agent. *Plant Dis* **80**, 1203–1206.

Di Serio, F., Alioto, D., Ragozzino, A. & Flores, R. (1998). Both the small circular RNAs and the double-stranded RNAs associated with the chlorotic rusty spot disease of sweet cherry are also found in sour cherry with similar symptoms. *Acta Hortic* **472**, 291–297.

Ghabrial, S. A. (1998). Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* **16**, 119–131.

Ghabrial, S. A. (2001). Fungal viruses. In *Encyclopedia of Plant Pathology*, pp. 478–483. Edited by O. Maloy & T. Murray. New York: Wiley.

Ghabrial, S. A. & Castón, J. R. (2004). *Chrysoviridae*. In *Eighth Report of the International Committee on Taxonomy of Viruses*. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & A. L. Ball. London: Elsevier/Academic Press (in press).

Ghabrial, S. A., Buck, K. W., Hillman, B. I. & Milne, R. G. (2004). *Partitiviridae*. In *Eighth Report of the International Committee on Taxonomy of Viruses*. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & A. L. Ball. London: Elsevier/Academic Press (in press).

Ihrmark, K. (2001). *Double-stranded RNA elements in the root rot fungus Heterobasidion annosum*. PhD thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Jiang, D. & Ghabrial, S. A. (2004). Molecular characterization of *Penicillium chrysogenum virus*: reconsideration of the taxonomy of the genus *Chrysovirus*. *J Gen Virol* **85**, 2111–2121.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA 2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Livieratos, I. C., Avgelis, A. D. & Coutts, R. H. A. (1999). Molecular characterization of cucurbit yellow stunting disorder virus coat protein gene. *Phytopathology* **89**, 1050–1055.

Oh, C.-S. & Hillman, B. I. (1995). Genome organization of a partitivirus from the filamentous ascomycete *Atkinsonella hypoxylon*. *J Gen Virol* **76**, 1461–1470.

Osaki, H., Nomura, K., Iwanami, T., Kanematsu, S., Okabe, I., Matsumoto, N., Sasaki, A. & Ohtsu, Y. (2002). Detection of a double-stranded RNA virus from a strain of the violet root rot fungus *Helicobasidium mompa* Tanaka. *Virus Genes* **25**, 139–145.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Strauss, E. E., Lakshman, D. K. & Tavantzis, S. M. (2000). Molecular characterization of the genome of a partitivirus from the basidiomycete *Rhizoctonia solani*. *J Gen Virol* **81**, 549–555.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.