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Analysis of Clinical Ostreid Herpesvirus 1 (*Malacoherpesviridae*) Specimens by Sequencing Amplified Fragments from Three Virus Genome Areas

Tristan Renault[a,*], Pierrick Moreau[a], Nicole Faury[a], Jean-François Pepin[a], Amélie Segarra[a] and Stephen Webb[b]

[a] Ifremer (Institut Français pour la Recherche et l'Exploitation de la Mer), Laboratoire de Génétique et de Pathologie, Ronce les Bains, La Tremblade, France
[b] Cawthron Institute, Nelson, New Zealand

*: Corresponding author : Tristan Renault, Tel. : 33 5 46 76 26 26 ; Fax: 33 5 46 76 26 11
email address : Tristan.Renault@ifremer

Abstract:

Although there are a number of ostreid herpesvirus 1 (OsHV-1) variants, it is expected that the true diversity of this virus will be known only after the analysis of significantly more data. To this end, we analyzed 72 OsHV-1 “specimens” collected mainly in France over an 18-year period, from 1993 to 2010. Additional samples were also collected in Ireland, the United States, China, Japan, and New Zealand. Three virus genome regions (open reading frame 4 [ORF4], ORF35, -36, -37, and -38, and ORF42 and -43) were selected for PCR analysis and sequencing. Although ORF4 appeared to be the most polymorphic genome area, distinguishing several genogroups, ORF35, -36, -37, and -38 and ORF42 and -43 also showed variations useful in grouping subpopulations of this virus.

Ostreid herpesvirus 1 (OsHV-1) has been classified within the *Malacoherpesviridae* family (6, 7, 12). Although OsHV-1 variants have already been reported (2, 3, 13, 19, 20), more work is needed to gauge the range of OsHV-1 polymorphisms. Since 2008, massive mortality outbreaks among Pacific oysters (*Crassostrea gigas*) have been reported in Europe (8, 21) associated with a virus genotype labelled µVar (22). In addition, mortality outbreaks were reported recently in New Zealand and Australia (15, 16) in association with a virus interpreted as OsHV-1 µVar. Moreover, a herpesvirus with close affinities to OsHV-1, the acute viral necrosis virus (AVNV), infecting Chinese cultured scallops *Chlamys farreri,* has been recently sequenced (GenBank accession n° GQ153938). Comparative genomic analysis between AVNV and OsHV-1 suggests that AVNV is a variant of OsHV-1 (Ren Weicheng, pers. com.).

Seventy-two samples of Pacific oysters collected from 1993 to 2010 and covering different stages of development (larvae, spat and adults) (Table 1) were selected. Most of the samples (a total of 63) were collected in France during episodes of mortality and were stored frozen at -20°C. Nine samples came from elsewhere (Ireland, China, Japan, USA and New Zealand) and included three paraffin-embedded archival specimens (Table 1). Nucleic acid extraction was performed by using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's handbook (17). For frozen tissues, 60-200 mg of larvae or 20-60 mg of mantle from juveniles and adults were used. For paraffin-embedded specimens (spat collected in 2005 during mortality events in New Zealand), five sections each of 30µm thickness were cut from each histology block (1, 4, 23).

PCR assays were performed using 3 extant primer pairs targeting 3 virus genome regions: C2/C6 (ORF4) (18), IA2/IA1 (ORFs 42/43) (22) and Del 36-37F2/Del 36-37R (5'-

ATACGATGCGTCGGTAGAGC-3’/5’-CGAGAACCCCATTCCTGTAA-3’) (ORFs 35/36/37/38) (Fig. 1). All tested samples yielded amplicons of expected sizes with primer pairs C2/C6 (709bp) and IA1/IA2 (607bp) except for one specimen, perhaps due to limiting template.(Fig. 2A). For the Del 36-37F2/Del 36-37R primer pair, DNA samples each gave 1 of 3 different patterns: a PCR product of expected size (989 bp), a PCR product of 384 bp, or no amplification (Fig. 2A). Twenty-eight French samples collected from 1993 to 2008 yielded 989 bp amplicons and might be interpreted as the reference type (accession n° AY509253), a virus isolated from French Pacific oyster larvae in 1995 (6). A large deletion (605 bp) was reported for all samples identified as being the variant OsHV-1 µVar (7 French samples collected in 2008, 13 French samples in 2009 and 2010 and a sample collected in Ireland in 2009), and also for samples collected in China, USA, Japan and New Zealand. Finally, a third group of virus specimens (from French oysters collected in 1993 and from 2003 to 2008) was defined based on the absence of amplification. This lack of amplification was not related to the absence of virus DNA, as amplicons were obtained from the same samples with the primer pairs C2/C6 and IA1/IA2 (Fig. 2A). The 605 bp deletion reported for OsHV-1 µVar and related specimens covered entirely both ORF36 and ORF37 and a part of ORF38 (Fig. 1). This deletion of 2 genes and the modification of a third might be more than coincidental with the apparently increased virulence of OsHV-1 µVar. ORF38 encodes a RING finger protein. The RING finger domain of ICP0 and homologs from alphaherpesviruses is required for the activation of quiescent genomes (5, 9, 10, 11, 14). Modifications of the RING finger protein encoded by ORF38 might affect its activities and influence OsHV-1 virulence.

For DNA samples extracted from paraffin-embedded specimens, PCR analyses were carried out using primer pairs C9/C10 (5'-GAGGGAAATTTGCGAGAGAA-3'/5'-ATCACCGGCAGACGTAGG-3'), CF/CR (5'-CCCCGGGGAAAAAGTATAAA-3'/5'-GTGATGGCTTTGGTCAAGGT-3') and Del 36-37F2/Del 36-37R (see above). C9/C10 and CF/CR primers targeted ORF4. Two histological blocks from the 3 analysed samples yielded amplicons with the 3 primer pairs used (Fig. 2B). For CF/CR primers, the 2 samples yielded a 157 bp amplicon similar in size to that obtained for OsHV-1 µVar (Fig. 2B). The specimen considered as the reference type (1995/020/France) gave a 173 bp product. For Del 36-37F2/Del 36-37R primer, a 384 bp amplicon was obtained for the 2 samples from New Zealand (Fig. 2B) although the specimen 1995/020/France (reference type) yielded a 989 bp product. The obtained PCR product sizes suggest that the variant OsHV-1 µVar or a related virus was present in Pacific oysters collected in New Zealand in 2005. Moreover, C9/C10 PCR products from a paraffin-embedded specimen appeared identical in sequence to OsHV-1 µVar presenting a single deletion in comparison with the sequence of OsHV-1 (accession n° AY509253) (data not shown).

PCR products were purified by kit (Amicon Ultra 0.5 ml 30K Centrifugal filter, Millipore) according to the supplied protocol and were then directly sequenced. Samples were loaded into ABI PRISM ® 3130 XL-Avant Genetic Analyzer. Phylogenetic analyses were performed on sequence concatenations of the 3 genome areas (1426 positions) using the Maximum Likelihood method with the program MEGA5 (24). Partial PCR product sequences from virus specimens were submitted to GenBank (Table 1).

IA1/IA2 amplicon sequences (ORFs 42/43) were compared to OsHV-1 (accession n° AY509253) using the ClustalW program. Two mutations were observed differentiating

104 samples in 3 groups: the first one containing samples presenting 100% identities with
105 OsHV-1 (accession n° AY509253, reference type), the second presenting 100% identities
106 with OsHV-1 µVar (22) and the third group contained both samples collected in New
107 Zealand in 2010 presenting only one of the mutations characterizing OsHV-1 µVar, an A
108 deletion (22).
109 For Del 36-37F2/Del 36-37R, amplicons from samples collected in France in 2009 and
110 2010, some of the samples collected in 2008 and samples collected in China, USA, Japan
111 and New Zealand demonstrated a 605 bp deletion. Although amplicons obtained from
112 samples collected in France from 1993 to 2007 and some of the samples collected in 2008
113 did not demonstrate the 605 bp deletion, a few point mutations were reported
114 differentiating 3 groups, one of these groups presenting 100% identity with the reference
115 type (accession n° AY509253).
116 Finally, C2/C6 amplicons demonstrated the highest polymorphism with 82 positions of a
117 460 nucleotide sequence showing mutations defining at least 19 virus groups. All the
118 French samples collected in 2009 and 2010 showed 100% identities with the OsHV-1
119 µVar sequence (accession n° HQ842610) except for 2 virus specimens (2010/02/France
120 and 2010/12/France). Although most of the French samples collected until 2007 were
121 similar to the reference type (accession n° AY509253), some of them demonstrated
122 mutations in comparison with this reference. Thus, polymorphisms for several
123 microsatellite zones with variable numbers of repetitions were observed: 5-9 A, 5-6 G, 4-
124 5 C and 4-13 CTA with 3 repetitions for AVNV, 4 for OsHV-1 µVar and 8 for the
125 reference type (data not shown). For this microsatellite zone, some virus specimens
126 collected in France showed also 9, 11 or 13 repetitions.

Phylogenetic analysis allowed identification of 2 main groups from 54 virus specimens (Fig. 3). A first group contained French specimens collected from 1994 to 2008 including the reference type (accession n° AY509253). This group also integrated samples collected in USA and in China, and AVNV (Fig. 3). The second main group was composed of French specimens collected from 2008 to 2010 and an isolate collected in Ireland in 2009. The sequence of OsHV-1 µVar deposited in GenBank (accession n° HQ842610) was included in this group. It also integrated samples from Japan and New Zealand (Fig. 3). Although the C2/C6 fragment sequence for these specimens (Japan and New Zealand) was similar to OsHV-1 µVar sequence (accession n° HQ842610), they differed from HQ842610 by two shared mutations (two A replaced by two G).

Several French samples collected from 1993 to 2008 demonstrated 100% identities with the reference type and as such could be identified as OsHV-1 (6). Other samples collected in France from 2003 to 2008 showed some differences in comparison with the reference type. Although they appeared closely related to this virus type (accession n° AY509253) for ORF4, these samples did not yield amplicons when the Del 36-37F2/Del 36-37R primer pair was used. These results suggest that different OsHV-1 variants coexisted in France before 2008. The variant OsHV-1 µVar was not detected in French samples collected before 2008 in the present study. These results are in accordance with those reported by Segarra et al. (2010), who concluded that OsHV-1 µVar was not detected in archival samples and that, in Europe, OsHV-1 µVar was an emerging genotype. Phylogenetic analysis suggested also that although the reference type and OsHV-1 µVar share a common ancestor (Fig. 3), OsHV-1 µVar is not directly derived from the reference type. Moreover, 3 different types were detected in 2008 in France: specimens

identical to the reference type, viruses related to the reference type (closely related by the ORF4, but not amplified with Del 36-37F2/Del 36-37R primer pair) and specimens identical to OsHV-1 µVar. Finally, 2 French virus specimens collected in 1993 presented high homologies with the variant OsHV-1 Var (3). This variant was reported in 1997 during a mortality outbreak affecting both larval Pacific oysters and larval Manila clams, *Ruditapes philippinarum*, in a commercial hatchery (19, 20). Both French samples from 1993 correspond to *C. gigas* larvae collected in a commercial hatchery. It is thus possible that intensive farming conditions under which different bivalve species are kept at the same time in close proximity might promote interspecies transmission (2, 3).

The sample collected in California demonstrated the 605 bp deletion observed in the ORFs 35/36/37/38 area for OsHV-1 µVar. However, it did not present the mutations characterising this variant within the ORF4 and it appeared identical to OsHV-1 for the ORFs 42/43 area. AVNV grouped with the isolate collected in California based on sequence data obtained in the present study (Fig. 3). Although AVNV presents variations in coding and non-coding regions in comparison to OsHV-1, these results suggest strongly that AVNV is an OsHV-1 variant.

ORF4 appeared as the most polymorphic genome area distinguishing several genogroups. However, ORFs 35/36/37/38 and ORFs 42/43 areas also showed variations useful in defining different genotypes.

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Figure legends

Figure 1. Scale diagram of ORFs 35, 36, 37 and 38 for the OsHV-1 reference (A) and virus specimens presenting a 605 bp deletion (B) area. The vertical lines indicate the limits of the different ORFs. The deleted sequence is located between the dashed arrows: ORFs 36 and 37 are totally missing, as is a part of ORF38.

Figure 2A. PCR products (Del 36-37F2/Del 36-37R, C2/C6 and IA1/IA2) from selected virus specimens electrophoresed on 1.5% agarose. M: Small Marker (Eurogentec), 1: 1993/012/France, 2: 1994/005/France, 3: 1995/020/France, 4: 2005/012/France, 5: 2006/005/France, 6: 2007/004/France, 7: 2008/020/France, 8: 1993/002/France, 9: 2005/005/France, 10: 2006/009/France, 11: 2006/013/France, 12: 2007/028/France, 13: 2008/019/France, 14: 2008/073/France, 15: 2008/011/France, 16: 2010/001/New Zealand, 17: 2010/158 144/Japan, 18: 2009/002/France, 19: 2009/022/France, 20: 2010/002/France, 21: 2010/021/France and N: negative control.

Figure 2B. PCR analysis of the DNA samples extracted from histological blocks (Pacific oysters collected in 2005 in New Zealand during a mortality outbreak). M: Small Marker (Eurogentec), 1: 2005/2E/New Zealand, 2: 2005/2H/New Zealand, 3: 1995/020/France (OsHV-1 reference), 4: 2008/055/France (OsHV-1 µVar) and N: Negative control.

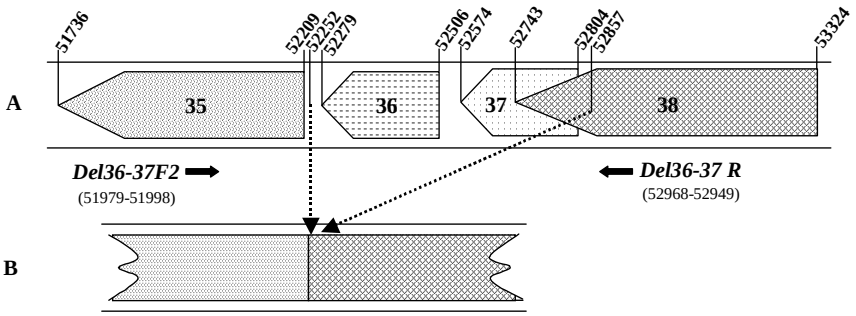
Figure 3. Phylogenetic tree generated by the Maximum Likelihood method. Bootstrap values were obtained from 1 000 resampled datasets. The analysis involved 54 nucleotide sequences (C2/C6, Del 36-37F2/Del 36-37R and IA1/IA2 concatenated PCR products). There were a total of 1426 positions in the final dataset. OsHV-1

(reference type) and AVNV were also included for phylogenetic analysis.

Table

Table 1. List of samples: DNA extracted from *C. gigas*, geographical origins, year of sampling, stage of development and GenBank accession numbers

273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52209
52252
52279
52506
52574
52743
52804
52857
53324
A
35
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38
Del36-37F2
(51979-51998)
Del36-37 R
(52968-52949)
B

Figure 1

274

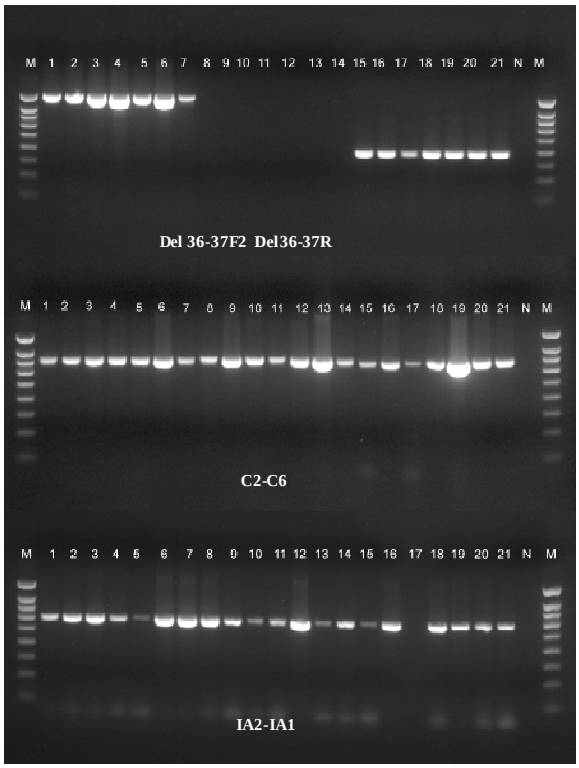


Figure 2A

275

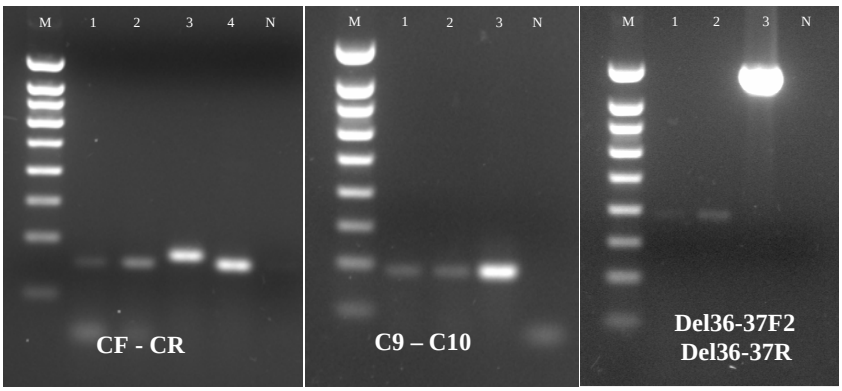
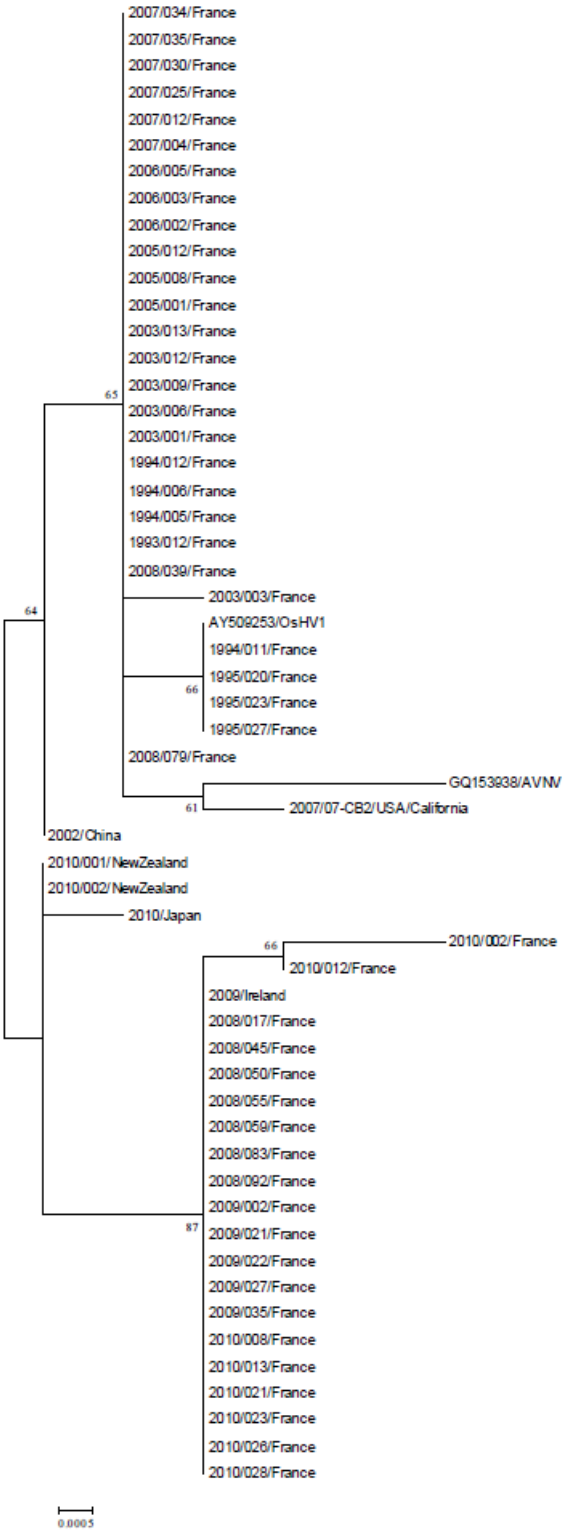


Figure 2B

276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2003/001/France
1994/012/France
1994/006/France
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1993/012/France
2008/039/France
2003/003/France
64
AY509253/OsHV1
1994/011/France
1995/020/France
66
1995/023/France
1995/027/France
2008/079/France
GQ153938/AVNV
61
2007/07-CB2/USA/California
2002/China
2010/001/NewZealand
2010/002/NewZealand
2010/Japan
66
2010/002/France
2010/012/France
2009/Ireland
2008/017/France
2008/045/France
2008/050/France
2008/055/France
2008/059/France
2008/083/France
2008/092/France
2009/002/France
87
2009/021/France
2009/022/France
2009/027/France
2009/035/France
2010/008/France
2010/013/France
2010/021/France
2010/023/France
2010/026/France
2010/028/France
0.0005

Figure 3

Table

Table 1 List of isolation codes of DNA extracted from *C. gigas* samples, geographical origins, years of sampling, stages of development, and GenBank accession numbers

Isolate code	Geographical origin of isolate	Yr of sampling	Development stage or age of isolate source	GenBank accession no. for sequence obtained with indicated primer pair		
				C2 and C6	Del 36-37F2 and Del 36-37R	IA1 and IA2
1993/002	France	1993	Larval	JN80065		JN800134
1993/004	France	1993	Larval	JN80066		JN800135
1993/012	France	1993	<1 yr	JN80067	JN800201	JN800136
1994/005	France	1994	<1 yr	JN80068	JN800202	JN800137
1994/006	France	1994	<1 yr	JN80069	JN800203	JN800138
1994/011	France	1994	<1 yr	JN80070	JN800204	JN800139
1994/012	France	1994	Larval	JN80071	JN800205	JN800140
1995/020	France	1995	Larval	JN80072	JN800205	JN800141
1995/023	France	1995	Larval	JN80073	JN800206	JN800142
1995/027	France	1995	Larval	JN80074	JN800207	JN800143
2003/001	France	2003	<1 yr	JN80075	JN800208	JN800144
2003/003	France	2003	<1 yr	JN80076	JN800209	JN800145
2003/006	France	2003	<1 yr	JN80077	JN800210	JN800146
2003/009	France	2003	<1 yr	JN80078	JN800211	JN800147
2003/012	France	2003	<1 yr	JN80079	JN800212	JN800148
2003/013	France	2003	<1 yr	JN80080	JN800213	JN800149
2005/001	France	2005	<1 yr	JN80081	JN800214	JN800150
2005/005	France	2005	<1 yr	JN80082	JN800215	JN800151
2005/008	France	2005	Larval	JN80083	JN800216	JN800152
2005/012	France	2005	<1 yr	JN80084	JN800217	JN800153
2006/002	France	2006	Larval	JN80085	JN800218	JN800154
2006/003	France	2006	Larval	JN80086	JN800219	JN800155
2006/005	France	2006	Larval	JN80087	JN800220	JN800156
2006/009	France	2006	<1 yr	JN80088		JN800157
2006/013	France	2006	<1 yr	JN80089		JN800158
2006/018	France	2006	<1 yr	JN80090		JN800159
2007/004	France	2007	Larval	JN80091	JN800221	JN800160
2007/012	France	2007	Adult	JN80092	JN800222	JN800161
2007/025	France	2007	<1 yr	JN80093	JN800223	JN800162
2007/026	France	2007	<1 yr	JN80094		JN800163
2007/028	France	2007	<1 yr	JN80095		JN800164
2007/029	France	2007	<1 yr	JN80096		JN800165
2007/030	France	2007	<1 yr	JN80097	JN800224	JN800166
2007/034	France	2007	1-2 yr	JN80098	JN800225	JN800167
2007/035	France	2007	<1 yr	JN80099	JN800226	JN800168
2008/017	France	2008	<1 yr	JN800100	JN800227	JN800169
2008/019	France	2008	1-2 yr	JN800101		JN800170
2008/021	France	2008	Larval	JN800102		JN800171
2008/023	France	2008	Adult	JN800103		JN800172
2008/025	France	2008	Adult	JN800104		JN800173
2008/030	France	2008	Adult	JN800105		JN800174
2008/039	France	2008	Larval	JN800106	JN800228	JN800175
2008/045	France	2008	1-2 yr	JN800107	JN800229	JN800176
2008/050	France	2008	<1 yr	JN800108	JN800230	JN800177
2008/055	France	2008	<1 yr	JN800109	JN800231	JN800178
2008/059	France	2008	<1 yr	JN800110	JN800232	JN800179
2008/073	France	2008	1-2 yr	JN800111		JN800180
2008/079	France	2008	<1 yr	JN800112	JN800233	JN800181
2008/083	France	2008	<1 yr	JN800113	JN800234	JN800182
2008/092	France	2008	<1 yr	JN800114	JN800235	JN800183
2009/002	France	2009	<1 yr	JN800115	JN800236	JN800184
2009/021	France	2009	<1 yr	JN800116	JN800237	JN800185
2009/022	France	2009	<1 yr	JN800117	JN800238	JN800186
2009/027	France	2009	<1 yr	JN800118	JN800239	JN800187
2009/035	France	2009	<1 yr	JN800119	JN800240	JN800188
2010/002	France	2010	<1 yr	JN800120	JN800241	JN800189

(Continued on following page)

TABLE 1 (Continued)

Isolate code	Geographical origin of isolate	Yr of sampling	Development stage or age of isolate source	GenBank accession no. for sequence obtained with indicated primer pair		
				C2 and C6	Del 36-37F2 and Del 36-37R	IA1 and IA2
2010/008	France	2010	<1 yr	JN800121	JN800242	JN800190
2010/012	France	2010	<1 yr	JN800122	JN800243	JN800191
2010/013	France	2010	<1 yr	JN800123	JN800244	JN800192
2010/021	France	2010	<1 yr	JN800124	JN800245	JN800193
2010/023	France	2010	<1 yr	JN800125	JN800246	JN800194
2010/026	France	2010	<1 yr	JN800126	JN800247	JN800195
2010/028	France	2010	<1 yr	JN800127	JN800248	JN800196
2002/E50	China	2002		JN800132	JN800253	
2007/07-CB2	USA (California)	2007		JN800128	JN800249	JN800197
2010/158-144	Japan	2010	<1 yr	JN800133	JN800254	
2009/Ireland	Ireland	2009		JN800129	JN800250	JN800198
2010/01	New Zealand	2010	<1 yr	JN800130	JN800251	JN800199
2010/02	New Zealand	2010	Larval	JN800131	JN800252	JN800200
2005/2E[a]	New Zealand	2005	<1 yr			
2005/2H[a]	New Zealand	2005	<1 yr			
2005/BN1C[a]	New Zealand	2005	<1 yr			

[a] DNA extracted from histological blocks.