Degradation of rRNA in BM-N cells from the silkworm *Bombyx mori* during abortive infection with heterologous nucleopolyhedroviruses

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Cell lines derived from the silkworm, *Bombyx mori*, are only permissive for *B. mori* nucleopolyhedrovirus (NPV), with other NPVs generally resulting in abortive infection. Here, we demonstrate that RNA of *B. mori* BM-N cells undergoes rapid degradation through site-specific cleavage upon infection with NPVs from *Autographa californica* (AcMNPV), *Hyphantria cunea* (HycuMNPV), *Spodoptera exigua* (SeMNPV) and *Spodoptera litura* (SpltMNPV). No significant decreases in cellular RNA were observed in Ld652Y, Se301, S19, SpIm and S2 cells infected with AcMNPV or HycuMNPV, indicating the response is unique to BM-N cells. A transient expression assay using a cosmid library of the HycuMNPV genome demonstrated that HycuMNPV P143 is responsible for rRNA degradation, which was also detected in BM-N cells transfected with plasmids expressing the P143 proteins from AcMNPV, SeMNPV and SpltMNPV. These results indicate that *B. mori* evolved to acquire a unique antiviral immune mechanism that is activated by P143 proteins from heterologous NPVs.

INTRODUCTION

Nucleopolyhedroviruses (NPVs), which are members of the family *Baculoviridae*, are capable of entering a phylogenetically broad range of insect cells, including those that are non-permissive for viral replication (Katou et al., 2006, 2010; Miller & Lu, 1997; Morris & Miller, 1993). Upon viral entry, insect cells exhibit various responses to the virus infection depending on the specific NPV–insect cell combination, resulting in different types of abortive infections (Morris & Miller, 1993; Shirata et al., 1999) as well as productive infection. Certain NPV-infected insect cells undergo apoptosis (Chejanovsky & Gershburg, 1995; Dai et al., 1999; Ishikawa et al., 2003; Palli et al., 1996; Yanase et al., 1998) or global protein synthesis shutdown (Du & Thiem, 1997a, b; Guzo et al., 1991; Kamita & Maeda, 1993; Shirata et al., 2004, 2010), which both function as important antiviral defence mechanisms of lepidopteran insects (Clarke & Clem, 2003a; Clem, 2005; Ikeda et al., 2013; Thiem, 2009) and cause either abrogation or severe restriction of progeny virus production (Clarke & Clem, 2003b; Clem & Miller, 1993; Hershberger et al., 1992; Ishikawa et al., 2004).

Global protein synthesis shutdown has been observed in several NPV–insect cell systems (Ikeda et al., 2013; Thiem, 2009), including *Lymantria dispar* Ld652Y and *Bombyx mori* BmN-4 cells infected with *Autographa californica* multiple NPV (AcMNPV) (Du & Thiem, 1997a, b; Guzo et al., 1991; Kamita & Maeda, 1993), *Trichoplusia ni* TN368 cells infected with a recombinant AcMNPV defective in the hcf-1 (host cell factor 1) gene (Lu & Miller, 1996) and BmN-4 cells infected with *Hyphantria cunea* MNPV (HycuMNPV) (Shirata et al., 2004, 2010). In AcMNPV-infected *B. mori* cells, the virus-encoded p143 gene is involved in global protein synthesis shutdown and the substitution of one or two amino acid residues of AcMNPV P143 with the corresponding residue(s) of *B. mori* NPV (BmNPV) P143 precludes the abrogation of protein synthesis, leading to successful viral replication (Argaud et al., 1998; Crozier et al., 1994; Kamita & Maeda, 1997). Furthermore, rRNA levels are markedly decreased in BM-N cells infected with AcMNPV (Fujita et al., 2005).

In this study, we demonstrate that RNA of BM-N cells decreases rapidly upon infection with various heterologous NPVs, including HycuMNPV, AcMNPV, *Spodoptera exigua* MNPV (SeMNPV) and *Spodoptera litura* MNPV.
(SpltMNPV), while such decrease of cellular RNA does not occur in the BM-N cells infected with homologous BmNPV. Electrophoretic analysis revealed that extensive rRNA degradation occurs in the BM-N cells infected with heterologous NPVs. Our data also demonstrate that the rRNA degradation is not observed in cell lines S9, Ld652Y, Se301, Splm and S2 infected with AcMNPV or HycuMNPV, indicating that the RNA decrease and rRNA degradation resulting from NPV infection occur in a cell line-dependent manner. Transient expression assays using a cosmid library of HycuMNPV genome demonstrate that P143 proteins from heterologous NPVs are responsible for the rRNA degradation in BM-N cells.

RESULTS AND DISCUSSION

Site-specific cleavage and degradation of rRNA in BM-N cells infected with heterologous NPVs

BM-N cells were infected with either AcMNPV, HycuMNPV, SeMNPV, SpltMNPV or BmNPV, which replicate to high titres in BM-N cells, at multiplicities of infection (m.o.i.) of 10, 10, 1, 1 and 10 p.f.u. per cell, respectively. At 0, 4, 8 and 24 h post-infection (p.i.), total RNAs were isolated from infected cell lysates using a QIAcube instrument and RNeasy Mini kit, and were then quantified using a NanoDrop 2000 spectrophotometer. The total RNA content of BM-N cells decreased rapidly upon infection with AcMNPV, HycuMNPV, SeMNPV and SpltMNPV, whereas no significant decrease in RNA levels was observed in BmNPV-infected BM-N cells (Fig. 1a). In AcMNPV-infected BM-N cells, RNA started to decrease by 4 h p.i. and rapidly decreased thereafter to less than 45% at 8 h p.i. and 15% at 24 h p.i. of the level at 0 h p.i. Similar reductions in RNA levels during the first 24 h p.i. were also observed in BM-N cells infected with HycuMNPV, SeMNPV and SpltMNPV (Fig. 1a). These results indicate that rapid decrease in RNA in BM-N cells is caused not only by AcMNPV but also by various heterologous NPVs that are not productive in BM-N cells.

The total RNAs from BM-N cells infected with BmNPV, AcMNPV, HycuMNPV, SeMNPV or SpltMNPV (Fig. 1a) were analysed by electrophoresis using the MultiNA

![Fig. 1. Quantitative and electrophoretic analyses of total RNA isolated from BM-N cells infected with various NPVs. BM-N cells were infected with NPVs of B. mori (BmNPV), A. californica (AcMNPV), H. cunea (HycuMNPV), S. exigua (SeMNPV) and S. litura (SpltMNPV), and analysed at 0, 4, 8 and 24 h post-infection (p.i.). (a) Changes in total RNA in BM-N cells during NPV infection. Total RNAs were isolated from infected cells using a QIAcube instrument and RNeasy Mini kit, and were quantified based on the absorbance at 260 nm using a NanoDrop 2000 spectrophotometer. The error bars indicate the standard deviations of means from three determinations. (b) Changes in electrophoretic profiles of RNAs in BM-N cells during NPV infection. Electrophoretic analysis of RNAs was performed using the MultiNA system. Arrowheads to the right of the gel indicate 1500 nt (upper) and 1400 nt RNA bands (lower). Numbers to the left of the panel indicate RNA size markers in nt (RNA 6000 Ladder, Ambion).](http://vir.sgmjournals.org)
system. In BmNPV-infected BM-N cells, three major bands, two of which were considerably overlapped, with molecular sizes of ~2000 nt were observed, in addition to several faint bands (Fig. 1b). The electrophoretic profile of RNA from BmNPV-infected BM-N cells was similar to that of mock-infected BM-N cells (data not shown) and remained unchanged until 24 h p.i. The major three bands corresponded to the 18S rRNA and two 28S rRNA fragments generated by cleavage at the primary nick site (Applebaum et al., 1966; Greenberg, 1969; Ishikawa & Newburgh, 1972; Winnebeck et al., 2010). In contrast, in BM-N cells infected with AcMNPV, HycuMNPV, SeMNPV and SpltMNPV, the major rRNA bands of ~2000 nt decreased rapidly with increasing time p.i., and two minor RNA bands corresponding to molecular sizes of ~1500 and ~1400 nt were consistently detected at 8 h p.i. (Fig. 1b). At 24 h p.i., the major rRNA bands were markedly decreased and the 1500 nt band was not detected, whereas the 1400 nt band remained (Fig. 1b). The major rRNA bands steadily decreased with increasing times p.i. and were only barely detectable at 72 h p.i. (data not shown).

RNA decrease upon heterologous NPVs infection is cell line-dependent

To determine whether the decrease in RNA levels observed in BM-N cells also occurs in other insect cell lines in response to NPV infection, the cell lines Spodoptera exigua Se301, Ld652Y and Drosophila melanogaster S2, as well as Spodoptera frugiperda Sf9 and Spilosisma imparilis SpIm, which are conventional host cell lines of AcMNPV and HycuMNPV, respectively, were infected with AcMNPV and HycuMNPV at an m.o.i. of 10 p.f.u. Quantification of total RNAs during the first 24 h p.i. revealed that the RNA levels decreased markedly only in the control BM-N cells, and no significant decreases were observed in any of the other AcMNPV- or HycuMNPV-infected insect cell lines (Fig. 2). These results indicate that a decrease in cellular RNA upon infection with heterologous NPVs is an insect cell line-specific event.

P143s of heterologous NPVs are involved in the rRNA degradation in BM-N cells

To identify possible viral factor(s) responsible for the observed RNA degradation in BM-N cells, clones from a cosmid library of the HycuMNPV genome (Fig. 3a) were used to transfect BM-N cells, and at 72 h post-transfection (p.t.) total RNAs from the transfected cells were analysed by electrophoresis. Among the seven cosmids clones that covered the entire HycuMNPV genome, only BM-N cells transfected with clone hc-4 generated the 1400 nt rRNA fragment (Fig. 3d). Clone hc-4 DNA was double-digested with EcoRI and HindIII, and the six generated HycuMNPV genomic fragments (EE7.4K, HE6.7K, HH6.2K, HE5.8K, 4.3K and EE7.2K; Fig. 3b) were cloned into pBlueScript II KS + and then transfected into BM-N cells. However, none of the HycuMNPV genomic fragments encoded products that led to the cleavage or degradation of host cellular rRNA (Fig. 3e), suggesting that the viral gene(s) responsible for the rRNA cleavage and degradation was located in the gaps between the HycuMNPV genomic fragments (Ikeda et al., 2006).

To confirm this speculation, expression plasmids pPIGA3hr5/Hycu-p143, pPIGA3hr5/Hycu-VP39 and pPIGA3hr5/Hycu-GP41 (Fig. S1, available in JGV Online), which express Hycu-p143, Hycu-VP39 and Hycu-GP41, respectively, were transfected into BM-N cells. Only cells transfected with the plasmid harbouring hycu-p143 exhibited rRNA cleavage and degradation, as demonstrated by the weak, but clearly detectable, 1400 nt rRNA band (Fig. 3f). These results indicate that Hycu-P143 plays a role in triggering rRNA cleavage and degradation in BM-N cells.

To determine whether P143 proteins from other NPVs also triggered rRNA degradation in BM-N cells, the expression plasmids pPIGA3hr5/Ac-p143, pPIGA3hr5/Bm-p143, pPIGA3hr5/Se-p143 and pPIGA3hr5/Splt-p143 (Fig. S1), which express the P143 proteins of AcMNPV (Ac-P143), BmNPV (Bm-P143), SeMNPV (Se-P143) and SpltMNPV (Splt-P143), respectively, were transfected into BM-N cells.

![Fig. 2. Changes in total RNA levels in various insect cell lines during infection with AcMNPV and HycuMNPV. Se301, Sf9, SpIm, Ld652Y, S2 and BM-N cells were infected with either AcMNPV (black bars) or HycuMNPV (white bars) at an m.o.i. of 10 and at 0, 4, 8 and 24 h p.i., total RNAs were isolated and quantified. For details see the legend to Fig. 1.](image-url)
Electrophoretic analysis of total RNA from BM-N cells showed that all viral P143 proteins triggered rRNA cleavage and degradation, and led to the weak formation of the 1400 nt rRNA band (Fig. 4a). In addition, it was found that the BM-N cells transfected with each plasmid construct underwent severe apoptosis (Fig. 4b) that was accompanied by the activation of caspase-3-like protease (Fig. 4c). Notably, neither rRNA cleavage nor apoptosis was detected in BM-N cells transfected with the plasmid expressing Bm-P143 (Fig. 4). Taken together, these results suggest that in addition to rRNA cleavage and degradation, P143 proteins from heterologous NPVs trigger the apoptosis of BM-N cells.
rRNA degradation in BM-N cells by P143s from heterologous NPVs is not due to apoptosis

To exclude the possibility that the rRNA cleavage and degradation triggered by transiently expressed P143 proteins were associated with apoptosis induction, BM-N cells were transfected with pPIGA3hr5/Egfp or pPIGA3hr5/Ac-p143 and cultured in the presence and absence of a pancaspase inhibitor Z-VAD-FMK. The apoptosis triggered by transiently expressed Ac-P143 was suppressed by the Z-VAD-FMK treatment of transfected cells (Fig. 5a). In contrast, Z-VAD-FMK did not affect rRNA cleavage and degradation, and the 1400 nt rRNA band was clearly observed (Fig. 5b). These results demonstrate that the rRNA cleavage and degradation induced by heterologous NPVs in BM-N cells are independent of the apoptosis triggered by viral P143 proteins, and suggest that rRNA degradation and apoptosis are mediated by distinct signalling pathways, although they may share certain components.

Based on the above results, it is likely that viral P143 proteins play an important role in the rRNA cleavage and degradation observed in BM-N cells infected with heterologous NPVs. However, transient expression assays using plasmids encoding P143 proteins showed that rRNA cleavage and degradation were delayed by approximately 40 h and remained at lower levels compared to those in NPV-infected BM-N cells (Fig. 1b vs Fig. 4a). To further examine the role of P143 proteins in the cleavage and degradation of cellular rRNA, we constructed several AcMNPV bacmids that harboured p143 genes from heterologous, as well as homologous, NPVs, as described previously (Datsenko & Wanner, 2000). We first constructed an ac-p143-null bacmid (bAcp143KO) from bMON14272 (Invitrogen) and then inserted the coding regions of ac-p143, bm-p143, hycu-p143, se-p143 and splt-p143 into the polh locus of bAcp143KO under control of the ac-p143 promoter, generating bAcp143KO/ac-p143, bAcp143KO/bm-p143, bAcp143KO/hycu-p143, bAcp143KO/se-p143 and bAcp143KO/splt-p143.
respectively. The constructed AcMNPV bacmids were transfected into BM-N cells and total RNAs were analysed at intervals by MultiNA. The analysis revealed that all bacmids, with the exception of bAcp143KO/bm-p143 and bAcp143KO, triggered rRNA cleavage and degradation, and generated the 1400 nt rRNA fragment. However, the rRNA cleavage and degradation observed in the bacmid-transfected BM-N cells occurred more slowly and was less extensive compared to those in virus-infected BM-N cells (unpublished results). Accordingly, it is possible that early events

Fig. 5. Suppression of apoptosis by the pancaspase inhibitor Z-VAD-FMK in Ac-P143-expressing BM-N cells does not prevent rRNA cleavage and degradation. Monolayer cultures of BM-N cells (5×10⁵) in 35 mm culture dishes were transfected with 2 µg pPIGA3hr5/Egfp (EGFP) or pPIGA3hr5/Ac-p143 (Ac-P143) and cultured in 2 ml Sf-900II medium (Gibco) containing or not containing 50 or 100 µM Z-VAD-FMK. At 0 and 72 h post-transfection (p.t.), total RNAs were isolated and analysed by the MultiNA system as in Fig. 1. Z-VAD-FMK treatment was performed by adding 4 or 8 µl (final concentration: 50 or 100 µM, respectively) of a 25 mM Z-VAD-FMK stock solution in DMSO to 2 ml Sf-900II medium. For the controls, the same volumes of DMSO were added to the Sf-900II medium. (a) Microscopy of the BM-N cells at 72 h p.t. Scale bar, 50 µm. (b) Electrophoretic profiles of total RNAs at 0 and 72 h p.t. For details, see legend to Fig. 4.
specific to NPV-infected BM-N cells, but not bacmid-transfected BM-N cells, might enhance the rRNA cleavage and degradation triggered by P143 proteins. Comparative analyses using bacmids and viruses derived from the bacmid-transfected BM-N cells are currently under way in our laboratory to explore the discrepancy between virus-infected and bacmid-transfected BM-N cells.

The mechanism by which P143 proteins from heterologous NPVs trigger rRNA cleavage and degradation in BM-N cells is not known. The p143 gene is one of the core genes encoded in the genome of all baculoviruses sequenced to date (Herniou et al., 2003; van Oers & Vlak, 2007) and represents an early gene that is essential for viral DNA replication (Gordon & Carstens, 1984; Lu & Carstens, 1991). The p143 gene encodes a DNA helicase (Lu and Carstens, 1991; McDougal & Guarino, 2000) that is capable of binding both single- and double-stranded DNA (Laufs et al., 1997; McDougal & Guarino, 2000) and must be transported into the nucleus by LEF3 (late expression factor 3) to form a viral DNA replication complex that localizes in the virologic stroma (Chen & Carstens, 2005; Ito et al., 2004; Nagamine et al., 2006, 2011; Yu & Carstens, 2012) where viral DNA replication and morphogenesis occur. Three lines of evidence exclude the possibility that the rRNA degradation observed in heterologous NPV-infected BM-N cells was due to the viral DNA replication-related function of P143. First, among the heterologous NPVs that triggered rRNA degradation, SeMNPV and SpltMNPV are unable to replicate genomic DNA in infected BmN-4 cells (Lavina et al., 2001; Lavina-Caoili et al., 2001; Shirata et al., 1999; Wu et al., 2000). Second, our analysis with ts8, an AcMNPV mutant defective in viral DNA replication at non-permissive temperature (Lu & Carstens, 1991; Erlandson et al., 1984; Gordon & Carstens, 1984), demonstrated that rRNA cleavage and degradation occurred in the absence of viral DNA replication in ts8-infected BM-N cells cultured at a non-permissive temperature (unpublished results). Third, the cleavage and degradation of rRNA in NPV-infected BM-N cells is a very early event, being triggered by 4 h p.i., and therefore likely precedes the onset of viral DNA replication. Consequently, it is likely that a P143 protein-related event that occurs before the onset of viral DNA replication causes serious stress and triggers the degradation of rRNA in NPV-infected BM-N cells.

In conclusion, our present results demonstrate that B. mori cells have evolved a unique antiviral mechanism against baculovirus infection that is triggered by P143 proteins encoded by heterologous NPVs. The antiviral mechanism comprises apoptosis and rRNA degradation, which are likely linked to the global protein synthesis shutdown observed in B. mori cells infected with AcMNPV and HycuMNPV (Kamita & Maeda, 1993; Shirata et al., 2004, 2010). Thus, NPV-infected B. mori cells represent an excellent model system to study and gain a greater understanding of antiviral strategies of lepidopteran insects. Furthermore, in-depth characterization of the rRNA cleavage and degradation events in NPV-infected B. mori cells is expected to give evolutionary insight into the antiviral mechanisms underlying rRNA cleavage and degradation, including both RNase L-dependent (interferon-regulated 2-5A system) and RNase L-independent pathways, in virus-infected mammalian cells (Banerjee et al., 2000; Liang et al., 2006; Silverman, 2007; Sobol & Mossman, 2006).

METHODS

Cells, virus and infection. BM-N cells (Volkman & Goldsmith, 1982) from Bombyx mori, Sp9 cells (Vaughn et al., 1977) from Spodoptera frugiperda, Se301 cells (Hara et al., 1995) from Spodoptera exigua, IPL-B-Ld652Y (Ld652Y) cells from Lymantria dispar (Goodwin et al., 1978), S2 cells (Schneider, 1972) from Drosophila melanogaster, FRI-Splm-1229 (Splm) cells from Spilosoma imparilis (Mitsushashi & Inoue, 1988) were maintained as described previously (Ikedo et al., 2011). Viruses used in these experiments were Autographa californica M nucleopolyhedrovirus E2 (AcMNPV; Smith & Summers, 1978), Bombyx mori NPV N9 (BmNPV; Nagamine et al., 1989), Hyphantria cunea MNPV N9 (HycuMNPV; Kamiya et al., 2003), Spodoptera exigua MNPV G3 (SeMNPV; Wu et al., 2000) and Spodoptera litura MNPV P7 (SpltMNPV; Lavina et al., 2001).

These viruses were infected to the insect cells at varying input m.o.i. and allowed to adsorb to the cells for 60 min at room temperature as described previously (Shirata et al., 1999). Infected cells were washed three times with appropriate cell culture media, cultured in fresh medium and used for analysis at different times p.i. Time zero of infection was defined as the time when inoculum was removed from the cell culture.

Cosmid. Construction of the cosmid library of HycuMNPV genome has been described previously (Felipe Alves et al., 2002; Shirata et al., 2010). DNA fragments from the cosmid hc-4 double-digested with EcoRI and HindIII (see Fig. 3) were cloned into pBlueScript II KS+ vector (Stratagene) as described previously (Shirata et al., 2010).

Plasmids. Expression plasmid pPIGA3hr5/Egfp, which expresses EGFP, has been previously described (Fig. S1; Mitsutake & Kobayashi, 2011). Expression plasmids pPIGA3hr5/Ac-p143, pPIGA3hr5/Hycu-p143, pPIGA3hr5/Hycu-vp39, pPIGA3hr5/Hycu-gp41, pPIGA3hr5/Bm-p143, pPIGA3hr5/Bm-p143, pPIGA3hr5/Bm-p143 and pPIGA3hr5/Splt-p143 were constructed from pPIGA3hr5/Egfp. Linearized pPIGA3hr5/Egfp was obtained by inverse-PCR using the paired primers pPIGA3hr5-F and pPIGA3hr5-R (Table S1). The coding regions of hycu-p143, se-p143 and splt-p143 were PCR-amplified from the genomic DNAs of respective NPVs using specific primers which contain 12–15 bp extensions complementary to vector ends (Table S1, underlined):

Hycu-p143-F and Hycu-p143-R for hycu-p143, Se-p143-F and Se-p143-R for se-p143, Splt-p143-F and Splt-p143-R for splt-p143, and then fused with the linearized pPIGA3hr5/Egfp using In-Fusion HD cloning kit (Clontech). The coding regions of hycu-vp39, hycu-gp41, ac-p143 and bm-p143 were PCR-amplified from the genomic DNAs of respective NPVs using specific primers: Hycu-vp39-F and Hycu-vp39-R for hycu-vp39, and Hycu-gp41-F and Hycu-gp41-R for hycu-gp41, Ac/Bm-p143-F and Ac/Bm-p143-R for ac-p143 and bm-p143. The resulting PCR products were cloned into pCR4-TOPO (Invitrogen) to generate TOPO/Hycu-vp39, TOPO/Hycu-gp41, TOPO/Ac-p143 and TOPO/Bm-p143. The coding regions of hycu-vp39, hycu-gp41, ac-p143 and bm-p143 were excised from TOPO/Hycu-vp39, TOPO/Hycu-gp41, TOPO/Ac-p143 and TOPO/Bm-p143 by digestion with NcoI and NotI and then cloned into NcoI and NotI sites of pPIGA3hr5/Egfp to generate pPIGA3hr5/Hycu-vp39,
RNA extraction and electrophoretic analysis. At indicated times p.i., total RNAs were isolated from infected cell lysates using a QIAcube instrument (Qiagen) and RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions, and were then quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Electrophoretic analysis of RNA was performed by the microchip electrophoresis system using MultiNA (Shimadzu) according to the manufacturer’s instructions. Prior to the analysis, RNAs isolated from the NPV-infected cells were denatured at 65 °C for 5 min, followed by incubation on ice for 5 min.

Transfection. Transfection was performed as previously described (Ikedo et al., 2011). BM-N cells (5 × 10^5) in a 35 mm dish were transfected with 2 μg of plasmid using 4 μl of FuGene (Promega).

Apoptosis suppression. The transfected BM-N cells were cultured in 2 ml SF-900II medium (Gibco) containing or not containing 4 or 8 μl (final concentration: 50 or 100 mM, respectively) of 25 mM Z-VAD-FMK (Peptide Institute) in DMSO. For the controls, the same volumes of DMSO were added to the medium.

Caspase activity assay. Caspase-3-like protease activity in insect cells was determined as described previously (Ikeda et al., 2004; Ishikawa et al., 2003). The synthetic substrate Ac-DEVD-AMC (Peptide Institute) was used for the assay.

Nucleotide sequencing. Nucleotide sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and ABI 3130 Genetic Analyzer (Applied Biosystems), as described previously (Suganuma et al., 2011). The nucleotide sequences obtained were analysed using Genetyx software (Genetyx Corporation).

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