

***Boty-II*, a novel LTR retrotransposon in *Botrytis cinerea* B05.10 revealed by genomic sequence**

Ming Zhao

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China
Graduate University of Chinese Academy of Sciences
Beijing PR, China

Jin yan Zhou

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China

Zhi dong Li

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China

Wei wei Song

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China
Graduate University of Chinese Academy of Sciences
Beijing 100039, China

You jiu Tan

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China
Graduate University of Chinese Academy of Sciences
Beijing 100039, China

Hong Tan*

Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041, PR China
Tel: 86 028 85219314
Fax: 86 028 85219314
E-mail: abath@cib.ac.cn

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Abbreviations: INT: DDE integrase domain
LTR: long terminal repeat
N-J: neighbor-joining
PBS: primer binding site
PPT: polypurine tract
RT: reverse transcriptase domain
TE: transposable element
TSD: target-site duplications

***Botrytis cinerea* is a necrotrophic pathogen causing pre- and post-harvest diseases in at least 235 plant species. It manifests extraordinary genotype and phenotype variation. One of the causes of this variation is**

transposable elements. Two transposable elements have been discovered in this fungus, the retrotransposon (*Boty*), and the transposon (*Flipper*). In this work, two complete (*Boty-II-76* and *Boty-II-103*) and two partial

*Corresponding author

(*Boty-II-95* and *Boty-II-141*) long terminal repeat (LTR) retrotransposons were identified by an *in silico* genomic sequence analysis. *Boty-II-76* and *Boty-II-103* contain 6439 bp nucleotides with a pair of LTRs at both ends, and an internal deduced *pol* gene encoding a polyprotein with reverse transcriptase and DDE integrase domains. They are flanked by 5 bp direct repeats (ACCAT, CTTTC). In *Boty-II-141*, two LTRs at both ends, and a partial internal *pol* gene encoding a protein with a DDE integrase domain were identified. In *Boty-II-95*, a right LTR and a partial internal *pol* gene encoding a protein with no conserved domains were identified. *Boty-II* uses a self-priming mechanism to initiate synthesis of reverse transcripts. The sequence of the presumed primer binding site for first-strand reverse transcription is 5'-TTGTACCAT-3'. The polypurine-rich sequence for plus-strand DNA synthesis is 5'-GCCTTGAGCGGGGGGTAC-3'. Fourteen *Boty-II* LTRs that contain 125-158 bp nucleotides and share 69.1 ~ 100% identities with the short inverted terminal repeats of 5 bp (TGTC...TGACA) were discovered. Analysis of structural features and phylogeny revealed that *Boty-II* is a novel LTR retrotransposon. It could potentially be used as a novel molecular marker for the investigation of genetic variation in *B. cinerea*.

Botrytis cinerea Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), the cause of grey mould, is a necrotrophic pathogen causing pre- and post-harvest diseases in at least 235 plant species. It is an important pathogen of nursery plants, vegetables and ornamentals, as well as field and orchard crops, and stored and transported agricultural products (Elad et al. 2007). World wide losses from this fungus account for 20% of the harvest of the affected crops, and their cost is estimated at 10-100 billion euros per year (Genoscope, 2008). The market size for anti-*Botrytis* products has been 15-25 million US dollars in recent years (Elad et al. 2007).

B. cinerea has a reputation for unusual genetic diversity and morphological variability, since *B. cinerea* isolates have morphologically distinct cultural types and have a tendency to change constantly during successive generations *in vitro* or under field conditions. Subcultures from single conidia often differ in morphotype from the parents and from each other. The genotypic and phenotypic variation is thought to be important to the changes in populations that occur in response to the broad host plants and fungicides. Development of resistance to fungicides has been recorded in *Botrytis* populations throughout the history of the modern fungicide era (Beever and Weeds, 2007; Elad et al. 2007). The causes of the genotypic and phenotypic variation are thought to be sexual reproduction, heterokaryosis, aneuploidy and extrachromosomal elements like mitochondria, viruses, plasmids and transposable elements (Beever and Weeds, 2007).

Transposable elements (TEs) are mobile, repetitive DNA sequences of genomes that can alter the sequence,

expression and chromosome rearrangements of genes. These changes have the potential to cause wide-ranging genetic variation, giving populations the flexibility to adapt successfully to environmental conditions, particularly in species that do not have a sexual phase (de Lima Fávoro et al. 2005). TEs are grouped into two classes by their transposition intermediate: RNA (class I or retrotransposons) or DNA (class II or DNA transposons) (Wicker et al. 2007). In *B. cinerea*, two TEs have been found: a retrotransposon (*Boty*), and a transposon (*Flipper*) (Diolez et al. 1995; Levis et al. 1997). *Boty* contains 596 bp long terminal repeats (LTR) and one internal putative *gag-pol* gene that encodes a polyprotein with sequences homologous to the reverse transcriptase (RT) and RNase H (RH) domains of retroelement *pol* genes (Diolez et al. 1995). *Flipper* is 1842 bp long with perfect inverted terminal repeats of 48 bp and an open reading frame (ORF) encoding a transposase (Levis et al. 1997). The two TEs are important and useful molecular markers for the investigation of genetic diversity in *B. cinerea*. Based on the presence or absence of *Boty* and *Flipper*, isolates having both TEs (*transposa*), isolates without TE (*vacua*), isolates only containing *Boty*, and isolates only containing *Flipper* were identified (Giraud et al. 1997; Giraud et al. 1999; Muñoz et al. 2002; Ahmed and Hamada, 2005; Ma and Michailides, 2005; Milicevic et al. 2006; Kretschmer and Hahn, 2008).

The 4.5 x whole genome shotgun (WGS) sequences of *B. cinerea* strain B05.10 are publicly available (Billault et al. 2006). In this work, a LTR retrotransposon designated *Boty-II* was identified by *in silico* genomic sequence analysis. Its structural characteristics, including its short inverted terminal repeats, the length of its LTRs, and its primer binding site (PBS) and polypurine tract (PPT) sequences revealed that *Boty-II* is a novel LTR retrotransposon. This was validated by phylogenetic analyses based on LTR DNA sequence and RT domain amino acid sequences. *Boty-II* potentially provides a molecular marker for surveying the genetic diversity of *B. cineria*.

METHODS

Boty-like element analysis

The DNA sequence of the *Boty* LTR (GenBank Accession no: [X81790](#)) and internal *gag-pol* gene (GenBank Accession no: [X81791](#), (Diolez et al. 1995)) were obtained from GenBank, and were used to query the genome database of *B. cinerea* B05.10 by Blastn search provided by the Broad Institute, (Altschul et al. 1997). All Blast hits of E value < 10⁻⁴ were extracted using Browse Region provided by the Broad Institute. Blast hits flanked by a pair of *Boty* LTRs were considered *Boty* retrotransposons. Blast hits not flanked by *Boty* LTRs are potentially the *gag-pol* genes of novel retrotransposons.

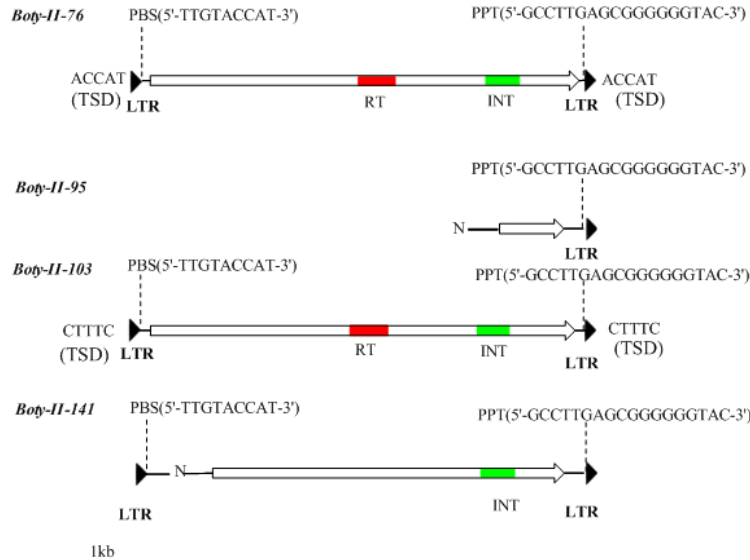


Figure 1. Diagram of *Boty-II*. DNA is depicted by —, Putative genes are depicted with the ⇨, LTRs are indicated with ✦, the putative reverse transcriptase domain (RT) is indicated with ■, the putative DDE integrase (INT) domain is indicated with ■, and N indicates gaps in the genomic sequence.

Novel LTR retrotransposon identification

To identify putative novel retrotransposons, we performed *in silico* chromosome walking in both directions on Blast hits not flanked by *Boty* LTRs. Approximately 1 kb up- and downstream of each Blast hit was extracted using Browse Region. The up- and downstream extracts of each Blast hit were aligned by bl2seq (<http://www.ncbi.nlm.nih.gov/Blast/bl2seq/wBlast2.cgi>), (Tatusova and Madden, 1999). The termini of alignments were analyzed by manual inspection. Elements flanked by short inverted terminal repeats, with high sequence similarities, were considered novel LTRs. Elements flanked by a pair of novel LTRs and containing internal deduced *gag-pol* gene were considered novel retrotransposons and designated as *Boty-II*.

Sequences of *Saccharomyces cerevisiae* tRNAs used for identification of PBS were obtained from the Genomic tRNA Database (Lowe and Eddy, 1997). The PPT and the target-site duplications (TSD) of LTR retrotransposon were investigated by manual inspection.

Distribution of *Boty-II* like elements

To identify the distribution of the *Boty-II* elements, the DNA sequence of one *Boty-II* was extracted using Browse Region, and used to query the *B. cinerea* B05.10 genome. All Blast hits were extracted and analyzed by manual inspection. Elements were designated *Boty-II-xx*, where xx is the supercontig number in which the *Boty-II* was detected. LTRs were designated *Boty-II-xx* L (R). Solo LTRs were named *S-II-xx*. For example, a novel LTR retrotransposon in the supercontig 76 was designated as

Boty-II-76, and the left (right) LTR of *Boty-II-76* was named *Boty-II-76- L (R)*.

Phylogenetic analyses

To verify *Boty-II* as a novel LTR retrotransposon, two phylogenetic analyses were developed. One was based on the nucleotide sequences of *Boty-II* and *Boty* LTRs, the other on the deduced amino acid sequences for the RT domains of *Boty-II* and *Boty*. Multiple alignments of protein or DNA sequences were created by MEGA version 4 (Tamura et al. 2007). Phylogenetic trees were constructed based on the alignment by MEGA version 4 using the neighbor-joining (N-J) method (Saitou and Nei, 1987). The tree was subjected to bootstrap resampling of 1000 replicates. Default parameters were accepted for all programs.

RESULTS AND DISCUSSION

Novel LTR retrotransposons in the genome of *B. cinerea* B05.10

Using the *Boty* LTR and internal *pol* gene sequence as queries, 27 putative *gag-pol* genes were identified and 23 of them were flanked by *Boty*-like LTRs. Four genes showed homology to the *gag-pol* retrotransposon gene, and were not flanked by *Boty* LTRs, indicating novel LTRs retrotransposons in *B. cinerea* B05.10 genome. We performed *in silico* chromosome walking in both directions on Blast hits that were not flanked by *Boty* LTRs. Approximately 1 kb up- and downstream of each Blast hit was extracted, and aligned by bl2seq. A novel type of LTR, designated *Boty-II* LTR, was identified. Elements that contained putative internal *gag-pol* genes and were flanked

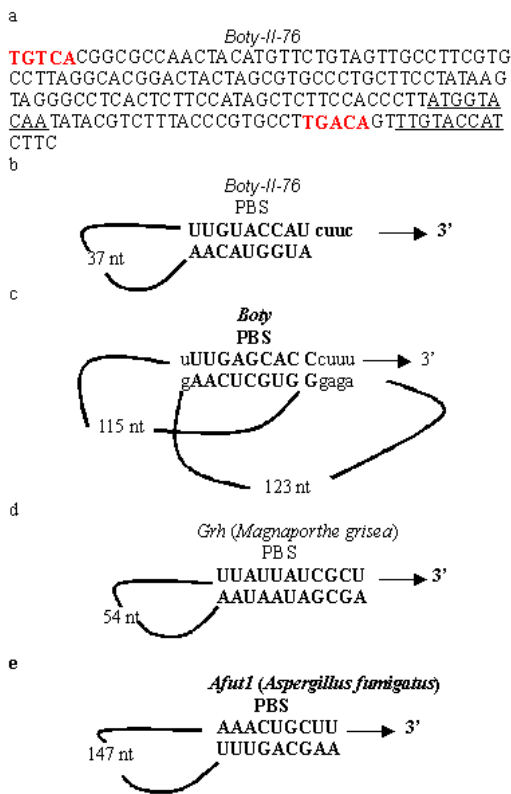


Figure 2. a DNA sequences of *Boty-II-76* left LTR and PBS. Short inverted terminal repeats are indicated in red, the PBS and nucleotides complementary to LTR are underlined; b, c, d, e Putative RNA structures of *Boty-II-76*, *Boty*, *Grh* and *Afut1*. The names of TR-retrotransposons and host organisms are listed on the top of each structure. The position of the PBS is indicated by capitalization. The GenBank Accession numbers of retrotransposons used in the sequence analysis are *Afut1*, L76086; *Boty*, X 81790 and *Grh*, M77661. c, d, e is from Lin and Levin (1997).

by *Boty-II* LTRs represented novel LTR retrotransposons and were designated *Boty-II*.

Structural characteristics of *Boty-II*

Four *Boty-II* retrotransposons were identified and designated *Boty-II-76*, *Boty-II-95*, *Boty-II-103* and *Boty-II-141*. *Boty-II-76* and *Boty-II-103* are flanked by 5 bp direct repeats (ACCAT, CTTTC) representing TSD. Both contain 6439 bp nucleotides, and share 99.7% identity. They contain an internal deduced *pol* gene encoding for a polyprotein with RT and DDE integrase (INT) domains (Figure 1, Table 1). Each element contains an identical LTR at both ends. The LTRs of *Boty-II-76* and *Boty-II-103* share 99.3% identity, with one site mutation. Because of gaps in the genome, only partial sequences of *Boty-II-95* (149 bp right LTR and a partial internal *pol* gene encoding for a protein without conserved domains) and *Boty-II-141* (two LTRs sharing 149 bp, with 96.6% identity and a partial internal *pol* gene encoding for a protein with an INT domain) were identified (Figure 1, Table 1). The fact that

the domains of *Boty-II* within the *pol* gene are arranged in the order RT and INT revealed that *Boty-II* belonged to the *Ty3/gypsy* group of retrotransposons.

Most LTR retrotransposons have a region adjacent t to the left LTR known as the minus-strand PBS, which is complementary to the 3' end of this tRNA (Kordiš, 2005). We screened the PBSs of *Boty-II* using the sequences of *S. cerevisiae* tRNAs, and no complementarity to any *S. cerevisiae* tRNAs or to the highly conserved CCA sequence found at the 3'end of all tRNAs was detected (Kordiš, 2005). This indicated that *Boty-II* potentially uses a self-priming mechanism to initiate synthesis of reverse transcripts. We found that *Boty-II-76*, *Boty-II-103* and *Boty-II-141* contain the identical PBSs for first-strand reverse transcription with 9 nt (5'-TTGTACCAT-3') immediately downstream of the left LTR that are complementary to the 9 nt nucleotides of the LTR (Figure 2a, Figure 2b). Many *Tf1* elements, including *Tf1*, *Tf2*, *MAGGY*, *Skippy*, *Grh*, *Afut1*, *Cf1* and *Boty* are able to “self-prime” reverse transcription (Levin, 1995; Levin, 1996; Lin and Levin, 1997; Butler et al. 2001). This work revealed that *Boty-II* belongs to the *Tf1* group. Though *Boty* and *Boty-II* may both be able to “self-prime” reverse transcription, the PBSs of *Boty-II* and *Boty* are different. According Lin and Levin (1997), the PBS of *Boty* can potentially form extended RNA structures (Figure 2c). However, no extended RNA structure could be identified in the PBS of *Boty-II*, similar to *Grh* and *Afut1* (Figure 2b-2e) (Lin and Levin, 1997).

The polypurine-rich sequence that corresponds to the primer binding site for plus-strand DNA (PPT) synthesis is located immediately upstream of the right LTR, and the sequence of PPT in *Boty-II-76*, *Boty-II-95*, *Boty-II-103* and *Boty-II-141* is 5'-GCCTTGAGCGGGGGGTAC-3'. It is different from *Boty*, which is 5'-AGGCTAAGAAGGGGATAG-3' (Diolez et al. 1995).

Distribution and Phylogenetic analysis of *Boty-II* LTRs

To survey the distribution of *Boty-II* LTRs in *B. cinerea* B05.10 genome, *Boty-II-76-L* was extracted and used to

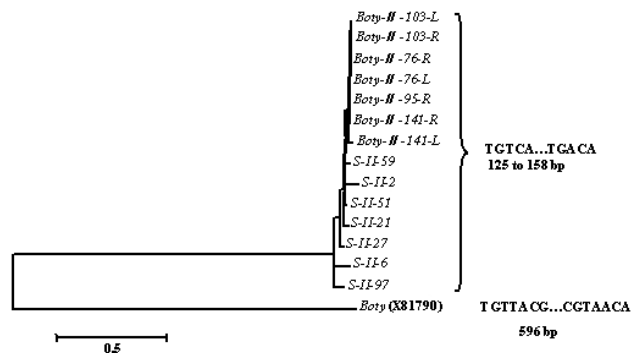


Figure 3. N-J tree based on alignment of *Boty* and *Boty-II* LTR DNA sequences.

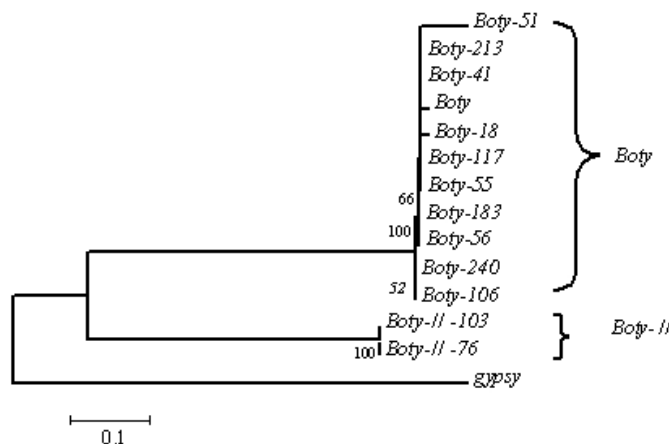


Figure 4. Phylogenetic analysis of LTR retrotransposons in *B. cinerea* based on alignment of the reverse transcriptase domains. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) and rooted using insect Gypsy LTR retrotransposons as outgroups. The number indicates the frequency with which a given branch appeared in 1000 bootstrap replications. Accession numbers for the elements in databases are as follows: *Boty*, [XP_001552885](#); *Boty-18*, [XP_001557158](#); *Boty-41*, [XP_001553972](#); *Boty-51*, [XP_001552885](#); *Boty-55*, [XP_001552584](#); *Boty-56*, [XP_001552482](#); *Boty-106*, [XP_001548698](#); *Boty-117*, [XP_001548080](#); *Boty-183*, [XP_001545956](#); *Boty-213*, [XP_001545506](#); *Boty-240*, [XP_001545348](#); *Boty-II-76*, [XP_001550688](#); *Boty-II-103*, [XP_001552861](#); gypsy, [AAA70219](#).

query the *B. cinerea* B05.10 genome. Fourteen *Boty-II* LTRs containing 125 to 158 bp nucleotides and sharing 69.1-100% identity were identified. They contained short inverted terminal repeats of 5 bp (TGTC...TGACA) (*Boty-II-103-L* and *Boty-II-103-R* contain TGTC...TGATA; *Boty-II-141-L* contains TGTC...TAACA; *S-II-27* contains TATCA...TGACA). The genome locations and structural features of *Boty-II* and *Boty-II* LTRs are in Table 1. *Boty* LTR contains 596 bp with a short, inverted 7 bp sequence (TGTTACG...CGTAACA) (Diolez et al. 1995). Phylogenetic analysis revealed *Boty* and *Boty-II* LTR form two clades (Figure 3). These results demonstrated that the *Boty-II* and *Boty* LTRs are different.

Phylogenetic analysis of LTR-retrotransposons in *B. cinerea*

To verify the differences in *Boty* and *Boty-II*, we developed a phylogenetic analysis of LTR-retrotransposons in *B. cinerea*. By Blast search, eleven *Boty* and two *Boty-II* elements containing the complete RT domains were identified. Amino acid sequences of RT domains were aligned and a N-J tree was constructed based on the alignment by MEGA4.0 (Tamura et al. 2007) (Figure 4). This phylogenetic analysis showed that *Boty* and *Boty-II* form two monophyletic clades, and validated *Boty-II* as a novel LTR retrotransposon in *B. cinerea*.

We concluded that *Boty-II* is a novel LTR retrotransposon in *B. cinerea* based on the following evidence: (1) *Boty-II* contains the internal *pol* gene flanked by LTRs, which is the hallmark of LTR retrotransposons; (2) *Boty-II* appears in multiple-copies in the *B. cinerea* B05.10 genome; (3) structural features of *Boty* and *Boty-II*, including the short inverted terminal repeats, the length of LTRs and the

sequences of PBS and PPT are different; (4) phylogenetic analyses based on DNA sequences of LTRs or amino acid sequences of RT domains revealed that *Boty* and *Boty-II* form two monophyletic clades.

Pathogen populations must constantly adapt to changes in their environment to survive. A plant pathogenic fungal population with a high level of genetic variation is likely to adapt more rapidly to fungicides or resistant host plants than populations with little or no genetic variation. Information on the genetic structure of this pathogen population can help us develop effective strategies for the control of disease (Váczy et al. 2007). Genetic variation in *B. cinerea* populations has been studied using resistance to botrycides and a variety of molecular techniques including restriction fragment length polymorphism (RFLP) analysis of PCR-amplified loci, PCR detection of TEs, random amplified polymorphic DNA (RAPD) fingerprinting, amplified fragment length polymorphism (AFLP) analysis, fingerprinting of repetitive sequences by microsatellite primed (MP)-PCR, PCR amplification of microsatellite loci and DNA sequencing of gene regions (Williamson et al. 2007). The DNA polymorphisms of nuclear genes showed that *B. cinerea* isolates consistently clustered in two different clades, Group I and Group II (Albertini et al. 2002; Fournier et al. 2002; Fournier et al. 2003; Fournier et al. 2005). Recently, analysis of microsatellite data showed that *B. cinerea* populations on grapevine and bramble were significantly differentiated, and indicated restricted gene flow, even in sympatry (Fournier and Giraud, 2008).

In this work, we report novel TEs and LTR retrotransposons (*Boty-II*) in *B. cinerea* B05.10. This potentially provides a novel molecular marker for the study of genetic variation in *B. cinerea*. Based on the presence or absence of the two known TEs in the genome, *B. cinerea*

has been grouped into four subpopulations (Giraud et al. 1997; Giraud et al. 1999; Muñoz et al. 2002; Ahmed and Hamada, 2005; Ma and Michailides, 2005; Milicevic et al. 2006). It will be interesting to survey the distribution of *Boty-II* in other isolates. We presume based on the presence or absence of the three TEs, *B. cinerea* will potentially be grouped into nine subpopulations.

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APPENDIX TABLE

Table 1. Summary of *Boty-II* elements in the *B. cinerea* B05.10 genome.

Names of <i>Boty-II</i> (Genome location)	Names and Short inverted terminal repeats of LTR (Genome location)	Putative internal protein (Genbank Accession numbers; Genome location)	Putative conserved domains of internal protein	Target site duplications
	<i>S-II-2</i> TGTCA...TGACA (Supercontig 2: 429990-430147 +)			ACTA
	<i>S-II-6</i> TGTCA...TGACA (Supercontig 6: 34252-34400 +)			
	<i>S-II-21</i> TGTCA...TGACA (Supercontig 21: 21: 668-816 +)			GAAAA
	<i>S-II-27</i> TATCA...TGACA (Supercontig 27 : 18469-18621 +)			
	<i>S -II-51</i> TGTCA...TGACA (Supercontig 51 : 163167-163291 +)			ATA
	<i>S-II-59</i> TGTCA...TGACA (Supercontig 59: 225815-225963 +)			TTATA
<i>Boty-II-76</i> (Supercontig 76: 146126-152564 +)	<i>Boty-II-76- L</i> TGTCA...TGACA (Supercontig 76: 146126-146274 +)	hypothetical protein BC1G_11096 (XP_001550688; Supercontig 76: 146446-152348+)	RT, INT	ACCAT
	<i>Boty-II-76- R</i> TGTCA...TGACA (Supercontig 76: 152416-152564 +)			
<i>Boty-II-95</i> (Supercontig 95: 159369-N*-)	<i>Boty-II-95-R</i> TGTCA...TGACA (Supercontig 95 : 159369-159517 -)			

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	<i>S-II-97</i> TGTC...TGACA (Supercontig 97 : 149630-149778 -)			CTACA
	<i>Boty-II-103-R</i> TGTC...TGATA (Supercontig 103: 52511-52659 -)			
<i>Boty-II-141</i> (Supercontig 141: 1246-6523 -)	<i>Boty-II-141-R</i> TGTC...TGACA (Supercontig 141: 193-337 -)	hypothetical protein BC1G_14556 (Supercontig 141: XP_001547118; Supercontig 141: 623- 2824)	INT	
	<i>Boty-II-141-L</i> TGTC...TGACA (Supercontig 141: 6375-6523 -)			

INT indicates DDE integrase domain; RT indicates reverse transcriptase domain; N indicates there are gaps in the genome.