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Simple and efficient recycling of fungal selectable marker genes with the Cre-*loxP* recombination system via anastomosis

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Abstract

Reverse-genetics analysis has played a significant role in advancing fungal biology, but is limited by the number of available selectable marker genes (SMGs). The Cre-*loxP* recombination system has been adapted for use in filamentous fungi to overcome this limitation. Expression of the Cre recombinase results in excision of an integrated SMG that is flanked by *loxP* sites, allowing a subsequent round of transformation with the same SMG. However, current protocols for regulated expression or presentation of Cre require multiple time-consuming steps. During efforts to disrupt four different RNA-dependent RNA polymerase genes in a single strain of the chestnut blight fungus *Cryphonectria parasitica*, we tested whether Cre could successfully excise *loxP*-flanked SMGs when provided *in trans* via anastomosis. Stable Cre-producing donor strains were constructed by transformation of wild-type *C. parasitica* strain EP155 with the Cre-coding domain under the control of a constitutive promoter. Excision of multiple *loxP*-flanked SMGs was efficiently achieved by simply pairing the Cre-donor strain and the *loxP*-flanked SMGs-transformed recipient strain and recovering mycelia from the margin of the recipient colony near the anastomosis zone. This method was shown to be as efficient as and much less time consuming than excision by transformation-mediated expression of Cre. It also allows unlimited recycling of *loxP*-flanked SMGs and the generation of disruption mutant strains that are free of any foreign gene. The successful application of this method to *Metarhizium robertsii* suggests potential use for optimizing reverse-genetics analysis in a broad range of filamentous fungi.

Keywords

selectable marker gene; Cre-*loxP* recombination; anastomosis; fungal transformation; *Cryphonectria parasitica*; gene knockout

1. Introduction

Advances in DNA sequencing technologies have resulted in the availability of genome sequence assemblies for an ever-growing number of filamentous fungi (e.g., Grigoriev et al.,

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2012). Functional analysis and genetic engineering of fungal genomes rely heavily on DNA-mediated transformation (Fincham, 1989). The efficiency of homologous transformation events has been improved significantly for an increasing number of fungal species by mutation of the nonhomologous end-joining (NHEJ) DNA-repair pathway (e.g., Ninomiya et al., 2004; Nayak et al., 2006; da Silva Ferreira et al., 2006; Krappmann et al., 2006; Takahashi et al., 2006; Poggeler and Kuck, 2006; Goins et al., 2006; Meyer et al., 2007; Ishibashi et al., 2006; Lan et al., 2008). However, the ability to disrupt multiple members of extensive gene families or to eliminate redundancy is restricted by the limited number of selectable marker genes (SMGs) that are available for filamentous fungi. This limitation has been addressed by the adaption of yeast (FLP/*FRT*) and bacteriophage (Cre-*loxP*) recombination systems to recycle SMGs (Kopke et al., 2010; Dennison et al., 2005; Florea et al., 2009; Forment et al., 2006; Krappmann et al., 2005; Patel et al., 2010). In the latter, more widely used recombination system, expression of an integrated Cre recombinase results in excision of an integrated SMG that is flanked by *loxP* sites, allowing a subsequent round of transformation with the same SMG lacking the flanking *loxP* sites.

Targeted gene disruption has been applied broadly in molecular studies on the chestnut blight fungus *Cryphonectria parasitica* that include investigations into signal transduction, virus-host interactions, fungal pathogenesis, RNA silencing and genetic non-self recognition (Reviewed in Nuss, 2005; Nuss, 2011 and Dawe and Nuss, In Press). Enhanced gene-disruption efficiency was recently achieved by disruption of the *ku80* nonhomologous end-joining DNA-repair pathway gene (Lan et al., 2008). The completion of a high-quality *C. parasitica* genome sequence assembly (http://genome.jgi-psf.org/Crypa2/Crypa2.home.html) by the Joint Genome Institute, Department of Energy, has provided opportunities to expand functional genomic analyses. In this regard, we recently adapted the Cre-*loxP* recombination system to recycle SMGs in an effort to disrupt four RNA-dependent RNA polymerase (*rdr*) genes in the same strain. Initial multistep protocols for providing Cre for excision of the *loxP*-flanked SMGs have been improved by use of inducible promoters (Krappmann et al., 2005; Forement et al., 2006), unselected transient Cre expression (Florea et al., 2009) and direct application of the Cre recombinase to fungal spheroplasts (Mizutani et al., 2012). In the course of conducting companion studies on the identification of vegetative incompatibility non-self recognition genes (Choi et al., 2012) that regulate the ability of *C. parasitica* strains to undergo anastomosis (hyphal fusion), we considered the possibility that Cre could be transferred via anastomosis for marker gene excision. Here, we describe an additional optimization for the use of the Cre-*loxP* recombination system in filamentous fungi by providing the Cre recombinase via anastomosis with a Cre-expressing donor strain. This method is efficient, requires less time and expense than current methods, allows unlimited recycling of *loxP*-flanked SMGs, and allows generation of disruption mutant strains free of any foreign genes. We also show that use of the method is not restricted to *C. parasitica* by demonstrating successful application to *Metarhizium robertsii*.

2. Materials and methods

2.1. Fungal strains and culture

The wild-type *C. parasitica* strain EP155 (ATCC 38755) was used to generate the Cre-expressing donor strain. Strain DK80, a mutant of EP155 disrupted in the *ku80* gene for NHEJ DNA repair to promote integration of homologous DNA sequence (Lan *et al.* 2008), was used to generate RNA-dependent RNA polymerase gene-disruption mutants. All *C. parasitica* cultures were maintained on PDA under 8 hours light at room temperature unless indicated otherwise. *Metarhizium robertsii* strain ARSEF 2575 (USDA/ARS Collection) was grown and maintained on PDA at room temperature.

2.2 DNA extraction and PCR conditions

Fungal genomic DNA for PCR was extracted as described by Spiering, et al. (2008). For PCR products smaller than 2 kb, PCR reactions were performed with AmpliTaq Gold polymerase (Life Technologies, Carlsbad, CA) using a 2-min extension time; for sizes larger than 3 kb, PCR reactions were performed with TakaRa ExTaq polymerase according to the manufacturer's manual (TaKaRa Clontech Laboratories, INC. Mountain View, CA).

2.3 Generation of RNA-dependent RNA polymerase gene-disruption mutants

RNA-dependent RNA polymerase genes were disrupted by replacing most of the *rdr*-coding sequences (CDSs) with selectable marker cassettes *via* homologous recombination in strain DK80 using the DNA transformation protocol developed by Churchill et al., (1990). Mutant strain Δ*rdr3* was generated with a split-marker approach (Fig. 1A). Two fragments of a *loxP*-pro$_{tubB}$-*hph*-*loxP* cassette [containing the promoter of the *tubB* gene from the fungus *Epichloë typhina* (Florea *et al.* 2009) to drive expression of *hph*] were PCR amplified from pKAES173, kindly provided by Dr. Chris Schardl, University of Kentucky. One fragment comprised 947 bp, corresponding to the first part of *loxP*-Pro$_{tubB}$-*hph*-*loxP*, and the second fragment contained 832 bp of the remaining cassette; the two fragments shared a 362-bp overlap within *hph*. The 5′ and 3′ *rdr3* flanking regions were then fused to each of the marker cassette fragments by overlap-extension PCR (primers used in this study are shown in Table 1), and both fragments were co-transformed into DK80 to generate Δ*rdr3* knockouts identifiable by *hph* resistance, indicating homologous recombination between the split marker fragments to give a functional *hph* gene.

To generate double knockouts, a neomycin (*neo*) gene cassette in pSK666 (Sun *et al.* 2009) was amplified with primer pair XbaIneo(d) and XhoIneo(u) and cloned into pKAES183 to replace Pro$_{tubB}$-phleomycin with the *neo* gene behind the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter from *A. nidulans*, resulting in a *loxP*-Pro$_{gpd}$-*neo*-*loxP* construct. Vector pKAES183 was derived from pKAES186 (provided by Dr. Chris Schardl, University of Kentucky) after XbaI digestion eliminating the fragment between two XbaI sites. Double knockouts were obtained by using the *loxP*-Pro$_{gpd}$-*neo*-*loxP* cassette to target a second *rdr* gene in the DK80-derived Δ*rdr3* mutant strain as shown for *rdr1* and *rdr2* in Figures 1B and 1C, respectively.

2.4 Construction of Cre-expression plasmid pCPX-cre and *C. parasitica* Cre-expressing donor strains

The *cre* gene was PCR amplified with primer pairs NotI-cre(d) and SphI-cre(u) from plasmid pKAES175 provided by Dr. Chris Schardl, University of Kentucky, and cloned between NotI and SphI sites into vector pCPX-*bsd1* (Suzuki *et al.*, 2003) behind the *gpd* promoter, resulting in the construct pCPX-cre with the *bsd* (blasticidin-S-deaminase) gene for selecting fungal transformants. Cre-expressing *C. parasitica* donor strains were produced by transforming *C. parasitica* wild-type strain EP155 with plasmid pCPX-cre. Blasticidin-resistant transformation candidates were taken through a round of asexual sporulation to ensure nuclear homogeneity (asexual spores are uni-nuclear) followed by PCR analysis with primer pair Pro-gpd(d) and SphI-cre(u) (Table 1) to confirm *cre* integration as indicated by a 2.6 kb amplicon.

2.5 Generation of a *Metarhizium robertsii* recipient strain containing a *lox*P-GFP/BAR-*lox*P expression cassette and a *M. robertsii* Cre donor strain

A *M. robertsii* strain 2575 expressing GFP from a *lox*P-GFP/BAR-*lox*P cassette was prepared by first replacing an XbaI-EcoRI fragment containing the bialaphos resistance (*bar*) cassette in plasmid pBAR (Fang et al., 2009) with two *lox*P-FRT repeats to create plasmid

pPK2H. GFP and *bar* expression cassettes were excised from pPK2SurGFPD (Lin et al., 2011) and pBARGPE1 (Pall and Bruhelli, 1993), respectively, and inserted in the polylinker (utilizing the BglII-EcoRI sites) region between the *lox*P-FRT repeats in plasmid pPK2H to create plasmid pPK2HBARGFP. A Cre expression plasmid pPK2Bar-Cre was prepared by first amplifying the Cre ORF from pCPX-cre with primers CRE-SmalF and CRE-EcoR1r (Table 1) and inserting the fragment into the corresponding sites between the *gpdA* promoter and *trpC* terminator in plasmid pBARGPE1 (Pall and Bruhelli, 1993) to form pBARGPE1-CRE. The Cre expression cassette was isolated following digestion with NdeI/SpeI from pBARGPE1-CRE, treated with T4 DNA polymerase and inserted into the EcoRV site of pBAR (Fang et al., 2009) to form pPK2Bar-CRE. Plasmids pPK2Bar-CRE and pPK2HBARGFP were used to transform *M. robertsii* strain 2575 to form the Cre donor strain and the *lox*P-GFP/BAR-*lox*P recipient strain, respectively, through *Agrobacterium tumefaciens*-mediated transformation as described by Fang et al., (2006). Successful transformation events were confirmed by PCR analysis with primer pairs cre(f)/cre(r) and GFP*lox*F/GFP*lox*R, respectively and by GFP fluorescence for pPK2HBARGFP transformants.

2.6 Transformation-mediated SMG excision in *C. parasitca*

For transformation-mediated excision, spheroplasts of *loxP*-flanked SMG transformed Δ*rdr* strains were transformed with the pCPX-cre plasmid, mixed into regeneration medium containing 600 μg/ml blasticidin and incubated for 7 days. Because of weak blasticidin selection, only larger colonies were chosen as putative transformants for further analysis. Putative transformats were sub-cultured on PDA or PDA amended with different antibiotics, depending on the SMGs being excised: PDA containing 50 μg/ml hygromycin or PDA containing 25 μg/ml G418 sulfate for *hph* or *neo*, respectively. Each plate contained multiple excision candidates along with strain DK80 (WT) as the SMG-negative control and the untreated Δ*rdr* mutant as the SMG-positive control. After 7 days growth, the candidate SMG-excised colonies exhibited poor to no growth on antibiotic plates, and the corresponding colonies of the candidates growing normally on antibiotic-free PDA were cultured and analyzed. Loss of the *loxP*-flanked *neo* and *hph* genes was confirmed by PCR, using the same primer pairs used to confirm *rdr* gene disruption (Figs. 1A-C; Table 1). Confirmation of SMG excision was followed by one round of single-sporing with antibiotic screening to ensure nuclear homogeneity.

2.7 Anastomosis-mediated SMG excision in *C. parasitica* and *M. robertsii*

For anastomosis-mediated excision, 1-mm agar plugs containing the Cre-expressing donor strain and a *loxP*-flanked SMG-transformed recipient strain were placed approximately 1-cm apart at the edge of 9-cm PDA plates. After 7-9 days of growth, mycelial plugs were recovered from the margin of the recipient colony at a point near the zone of contact between the two colonies and split into two subcultures. DNA was prepared from one of the subcultured colonies and tested for the presence of the excised SMG by PCR using the *rdr* primers for *C. parasitica* or the GFPloxf and GFPloxr primers (Table 1) for *M. robertsii*. Condia were collected from the second subcultured colony for those recipient mycelia that tested positive for excised PCR fragments and the single spore-derived colonies were screened for loss of antibiotic resistance for the *C. parasitca* recipient strains or for loss of GFP fluorescence for the *M. robertsii* recipient strain. Excision was further confirmed for single spore-derived colonies by PCR as described above followed by sequence analysis. The reproducibility and efficiency of the anastomosis-mediated excision method was examined by pairing multiple donor and recipient strains in multiple combinations, e.g., pairing of six independent *M. robertsii* Cre-donor strains with the same recipient strain and all available *C. parasitica* Δ*rdr* strains with a common Cre-donor strain.

3. Results

As part of ongoing investigations of the RNA-silencing antiviral defense response in *C. parasitica*, we identified four RNA-dependent RNA polymerase (*rdr*) gene candidates (manuscript in preparation). Although gene disruption is very efficient in the *C. parasitica ku80* NHEJ mutant strain DK80 (Lan et al., 2008), the disruption of multiple genes in one strain is challenging due to the limited number of selectable marker genes. Consequently, we employed the Cre-*loxP* recombination system to recycle SMGs. Selectable *loxP*-SMG-*loxP* cassettes containing hygromycin B (*hph*) or neomycin (*neo*) were used to disrupt the *rdr* genes as described in the Materials and methods (Fig. 1) and two Cre-mediated excision methods were tested.

3.1 Transformation of pCPX-cre into Δ*rdr1/3* and Δ*rdr2/3* successfully excised *loxP*-flanked *hph* and *neo* marker genes

We initially tested excision of *loxP*-flanked SMGs in *C. parasitica* by transformation of the disruption mutants with a Cre-expression plasmid, pCPX-cre. Blasticidin-resistant pCPX-cre transformants were then screened for loss of resistance to SMGs and excision was confirmed by PCR. As shown for the double-disruption mutant Δ*rdr2/3* in Fig. 2, following expression of Cre from the chromosomally integrated pCPX-cre plasmid both the *hph* and the *neo*-flanked *loxP* marker genes were efficiently excised from the disrupted *rdr2* (*neo*) and *rdr3* (*hph*) genes. In this case, ~80% of the selected pCPX-cre-Δ*rdr2/3* transformants failed to grow on 50 μg/ml hygromycin or 25 μg/ml of G418 and all tested transformants lacked both the *loxP*-flanked *neo* and *hph* marker genes. Similar effective excision was observed for pCPX-cre-transformed Δ*rdr1/3* strains (data not shown). While effective, this protocol is time consuming, requiring preparation of viable spheroplasts from the disruption mutants and selection of stable Cre-expressing and SMG-excised transformants. In addition, the excised mutant strains still retain the pCPX-cre plasmid for expression of Cre, thus preventing the use of *loxP*-flanked SMGs for subsequent gene disruption. A tightly controlled inducible promoter expression system is not currently available for *C. parasitica*.

3.2 Anastomosis-mediated SMG excision

As an alternative to providing the Cre recombinase by transformation, we considered the possibility that the Cre recombinase could effectively excise *loxP*-flanked SMGs when provided *in trans* from a Cre-expressing donor strain via anastomosis (fusion of hyphae). Anastomosis is a common process in most filamentous fungi (reviewed in Read et al., 2012) that readily occurs within colonies of the same strain or between strains that are not genetically different at genetic loci that regulate self recognition, the heterokaryon or vegetative incompatibility loci (Glass and Kuldau, 1992; Leslie, 1993). The protocol involves simply plating mycelial plugs of the *loxP*-flanked SMG-disruption mutant recipient strain the Cre-expressing donor strain 1 cm apart. As the colonies grow, they come into contact and form anastomoses along the zone of contact, allowing exchange of cytoplasmic materials, including transfer of the Cre recombinase to the recipient strain. Mycelial plugs are then recovered from the margin of the recipient strain colony just adjacent to the contact zone, slightly distal from the donor strain (Fig. 3). The recovered mycelia are subcultured, and tested for presence of excised SMG by PCR as described for the pCPX-cre transformants. Uninuclear condia derived from parallel cultures of the recipient mycelia that tested positive for excised SMGs by PCR are then cultured and screened for loss of antibiotic resistance.

Figure 4 shows the PCR characterization of recipient mycelia recovered from the pairing of a Cre donor strain in duplicate with the Δ*rdr3*, Δ*rdr1/3* and Δ*rdr2/3 lox*P-SMG-*lox*P recipient strains in which the *rdr3* gene was disrupted with *lox*P-*hph*-*lox*P and the *rdr1* and *rdr2* genes

were disrupted with *lox*P-*neo*-*lox*P. PCR analysis indicated the presence of excised *lox*P-*hph*-*lox*P fragments for the *rdr3* gene in all but one (Δ*rdr3#2*) recovered recipient mycelia and excised *lox*P-*neo*-*lox*P fragments for each of the recovered Δ*rdr1/3* and Δ*rdr2/3* recipient mycelia. The recovery of *lox*P-SMG-*lox*P free strains is readily accomplished by collecting uninuclear condia form the recovered recipient mycelia and selecting for antibiotic-sensitive colonies as shown for Δ*rdr2/3* in Figure 5A. PCR analysis is then used to confirm excision and the absence of non-excised *lox*P-SMG-*lox*P elements as shown in Fig. 5B for antibiotic sensitive single spore-derived colonies #2, #3, #4, #6 and #8 recovered from the screen shown in Fig. 5A. In parallel experiments, the pairing of four independent Cre-donor strains with available Δ*rdr* recipient strains was found to result in successful excision in each case (results not shown).

3.3 Anastomosis-mediated excision of *loxP*-GFP/BAR-*loxP* cassette from *M. robertsii*

To gain an indication of the general applicability of the anastomosis-mediated *cre*-excision method, we tested excision of a *lox*P-GFP/BAR-*lox*P cassette in the entomopathogenic fungus *Metarhizium robertsii*. This involved pairing of the *M. robertsii* Cre-expressing donor strain transformed with pPK2Bar-Cre with the recipient GFP producing/bar resistant strain transformed with plasmid pPK2HBARGFP to initiate anastomosis. Mycelia recovered from the margin of the recipient strain just adjacent to the donor strain were cultured and allowed to conidiate. Following PCR confirmation of the presence of *lox*P-GFP/BAR-*lox*P excision bands, condia form the corresponding recovered recipient colony were spread on PDA plates and examined by fluorescence microscopy for the loss of GFP expression. As an example, the presence of excised PCR bands was observed for each recovered recipient colony for the pairing of six independent *M. robertsii* Cre donor strains with the same *lox*P-GFP/BAR-*lox*P recipient strain. As shown in Fig. 6, both fluorescent and non-fluorescent colonies were recovered from the positive recipient strains. PCR analysis of DNA recovered from the non-fluorescent recipient colonies revealed only amplified fragments derived from the excised *lox*P-GFP/BAR-*lox*P cassette (results not shown).

Sequence analysis of the excision site fragments confirmed clean excision leaving only the 34 bases corresponding to the remaining unique *lox*P site sequence as shown in Fig. 7 for the excision of the *lox*P-flanked GFP/BAR cassette in the pPKH2BARGFP transformed *M. robertsii* (Fig. 7A), the *lox*P-flanked *neo* marker gene at the *C. parasitica rdr2* locus in a Δ*rdr2/3* recipient strain (Fig; 7B) and the *lox*P-flanked *hph* marker gene at the *rdr3* locus in the same Δ*rdr3* strain (Fig. 7C). Similar results were obtained for the *lox*P-flanked *neo* marker genes at the *rdr1* locus in Δ*rdr1/3* and for the l*ox*P-flanked *hph* marker gene at the *rdr3* locus in Δ*rdr3* and Δ*rdr1/3* (results not shown).

4. Discussion

We previously reported that anastomosis could be used to deliver a replication-dependent mycovirus-encoded protein, p48, to rescue p48-deficient, replication-defective viruses in a recipient *C. parasitica* strain (Deng and Nuss, 2008). The results presented here establish that anastomosis can be used as a simple method for delivery of the Cre recombinase for the efficient excision of *loxP*-flanked SMGs in filamentous fungui.

The anastomosis method has a number of advantages over existing protocols for Cre-mediated excision. There is no need to prepare spheroplasts of the *loxP*-flanked SMG transformed strain, nor for transient or stable transformation of the mutant with a Cre-expressing plasmid. In addition, multiple *loxP*-SMG-*loxP*-transformed lines can be paired simultaneously with the same Cre-expressing strain, little cost is involved and considerable time savings are achieved. Importantly, the anastomosis-mediated excision method allows unlimited recycling of *loxP*-flanked SMGs, unlike the case for transformation-mediated

excision where continued expression of Cre from the integrated Cre-expressing plasmid precludes subsequent recycling of *loxP*-flanked SMGs, unless tight inducible promoters, which are not currently available for many fungal species including *C. parasitica*, are used for Cre expression. Also, like the method of direct Cre delivery (Mizutani et al., 1993) or transient Cre-plasmid transfection (Florea et al., 2009), anastomosis-mediated Cre delivery results in the generation of disruption mutant strains that are free of any foreign gene. This is an important feature when considering regulatory issues pertaining to the release of fungi such as *C. parasitica* and *Metarhizium* species that have been engineered for enhanced biocontrol applications (Root et al., 2005; Fang et al., 2012). Finally, the successful use of this method to excise a *lox*P-flanked cassetee from *M. robertsii* shows that anastomosis-mediated *cre* excision is not limited to *C. parasitica* and predicts that the method may find broad application.

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Highlights

- Delivery of Cre recombinase by anastomosis excised fungal loxP-flanked marker genes.
- The method is time and cost saving.
- The method allows unlimited recycling of fungal marker genes.
- The method results in marker gene-free excision strains.
- The method was applied to *Cryphonectria parasitica* and *Metarhizium robertsii*.

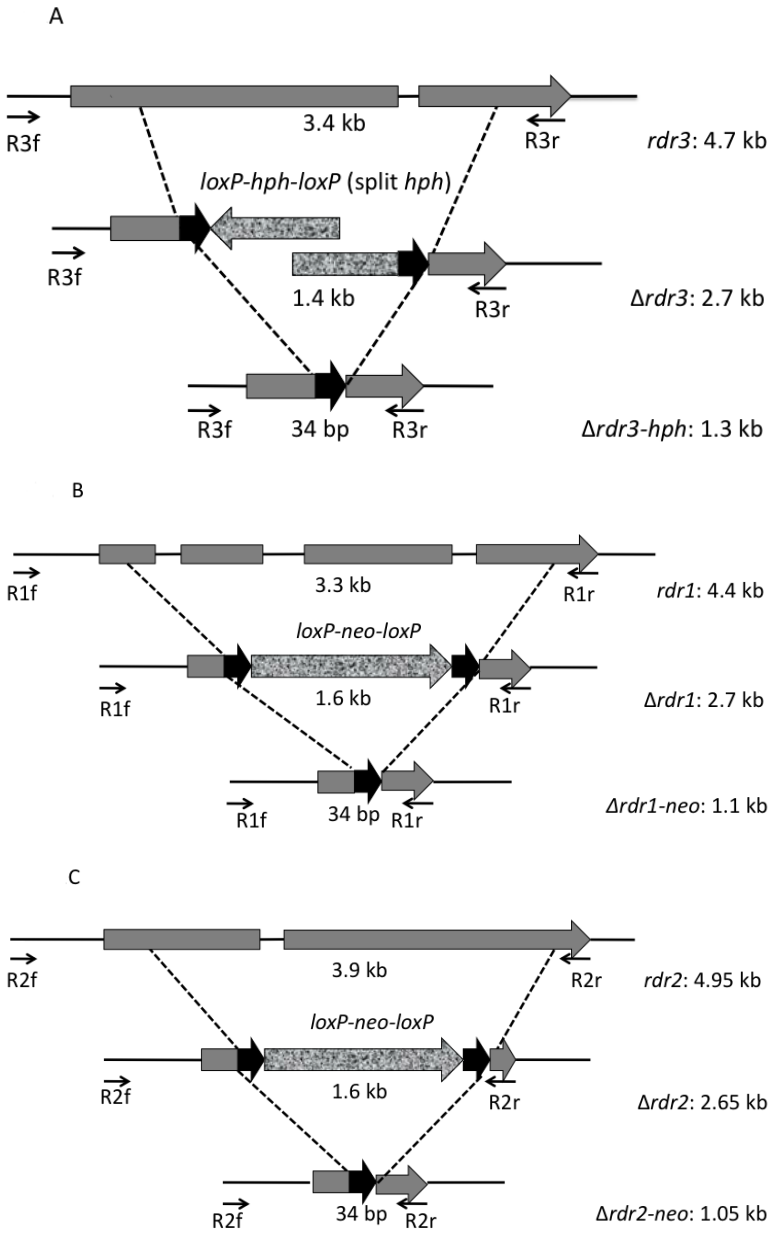

Figure 1.

C. parasitica RNA-dependent RNA polymerase (*rdr*) gene disruption mutants and SMG-excision products. (A). Disruption of *rdr3* involved use of the split-marker approach (Materials and methods) to replace a 3.4 kb portion of the *rdr3* CDS (spanning the region from 494 bp downstream of the start codon to 260 bp upstream of the stop codon), with a *loxP*-flanked *hph* cassette (1.4 kb). The solid grey boxes indicate the *rdr3* exons, while the thin line indicates the position of the single intron in *rdr3*. The *hph* resistance gene and *Epichloe typhina tubB* promoter cassette is indicated by the patterned shaded box. The thick black arrows indicate the locations of *loxP* sites, while the thin black arrows indicate the locations of the R3f and R3r *rdr3*-specific primers. The predicted sizes of PCR amplicons generated with the R3f/R3r primer pair for the wild-type (WT) *rdr3* gene, the disrupted *rdr3* gene (*Δrdr3*) and the SMG(*hph*)-excised disrupted *rdr3* gene (*Δrdr3-hph*, that retains a single *loxP* site) are shown at the right. The same conventions are used in panels B and C. (B).

Disruption of *rdr1* in the Δ*rdr3* mutant background to give Δ*rdr1/3* involved replacing a 3.3-kb portion of the *rdr1* CDS (232 bp downstream of the start codon and 204 bp upstream of the stop codon) with a 1.6-kb *loxP*-flanked G418 resistance (neomycin phosphotransferase) gene driven by the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene promoter (*loxP-neo-loxP*). (C). Disruption of *rdr2* in the Δ*rdr3* mutant background to give Δ*rdr2/3* involved replacing a 3.9 kb portion of the *rdr2* gene CDS (124 bp downstream of the start codon and 34 bp upstream of the stop codon) with the 1.6-kb *loxP-neo-loxP* cassette.

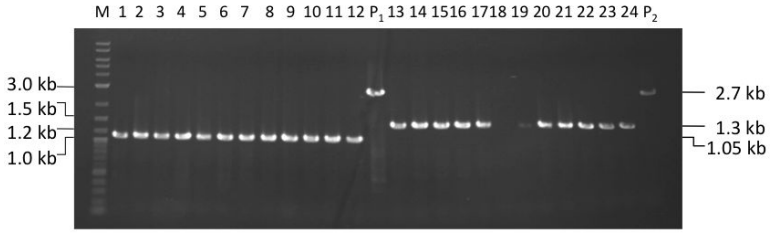

Figure 2.

PCR-agarose gel confirmation of excision of *loxP*-flanked *neo* and *hph* SMGs from a pCRX-cre-transformed Δ*rdr2/3* mutant strain. Twelve independent *bsd*-resistant pCRX-cre-transformed Δ*rdr2/3* colonies that grew slowly on *hph* and *neo* antibiotic plates were tested for excision of the *loxP*-flanked *neo* and *hph* SMGs from the disrupted Δ*rdr2* and Δ*rdr3* loci, respectively. Lane M contains DNA size markers with the corresponding kb sizes indicated at the left. Lanes P1 and P2 show migration positions of the amplicons generated when the *loxP*-flanked *neo* (2.65 kb) and *loxP*-flanked *hph* (2.7 kb) SMGs are intact, respectively. Lanes 1-12 contain the R2f/R2r primer pair-generated PCR amplicons from the 12 excision candidates showing the 1.05 kb band (position shown at right) expected if the *loxP*-flanked *neo* SMG was excised from the disrupted Δ*rdr2* gene locus. Lanes 13-24 contain the R3f/R3r primer pair-generated PCR amplicon from the same 12 excision candidates showing the 1.3-kb band (position at right) expected if the *loxP*-flanked *hph* SMG was excised from the disrupted Δ*rdr3* gene locus. Note that the PCR reaction for lane 18 failed.

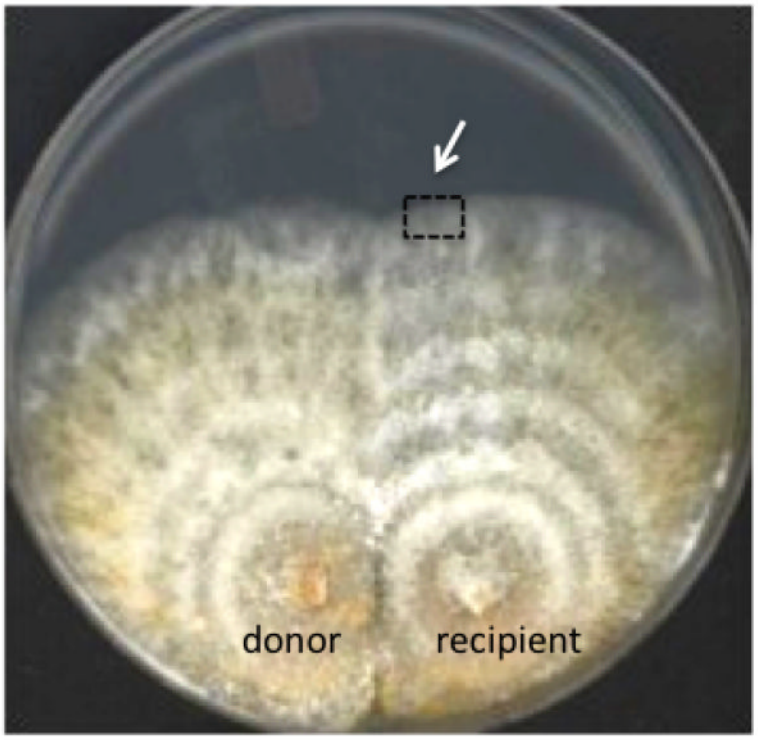

Figure 3.
Anastomosis-mediated SMG excision. This plate shows anastomosis pairing of a Cre-donor strain (left) and a recipient *loxP*-flanked SMG Δ*rdr*-disruption mutant strain (right). The colonies were incubated for 7 days after plating myclial plugs of the two strains 1 cm apart at the bottom of the plate. Mycelial plugs were removed from the area of the recipient strain colony just above the contact zone (boxed area), as indicated by the arrow, and analyzed for SMG excision.

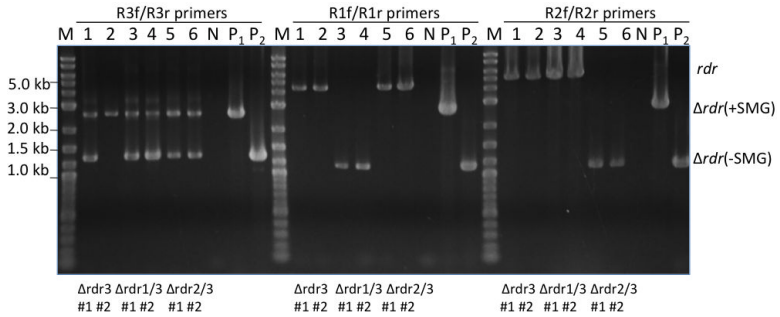

Figure 4.

PCR-agarose gel analysis of anastomosis-mediated excision of *loxP*-flanked *hph* and *neo* SMGs from *C. parasitica* Δ*rdr* recipient strains prior to single spore isolation. Lane M contains the DNA marker ladder with sizes in kb indicated at the left, while the position of specific amplicons corresponding to intact *rdr* genes [*rdr*], SMG-containing disrupted *rdr* genes [Δ*rdr*(+SMG)] and SMG-excised Δ*rdr* genes [Δ*rdr*(−SMG)] are indicated at the right. The primers used for PCR amplification are indicated at the top of each panel with the left most panel showing amplification of the *rdr3* locus (primers R3f/R3r), the middle panel showing amplification of the *rdr1* locus (primers R1f/R1r) and the right panel showing amplification of the *rdr2* locus (primersR2f/R2r). The recipient strains analyzed are indicated at the bottom of each panel with results for Δ*rdr3#1* and *#2* shown in lanes 1 and 2, results for Δ*rdr1/3#1* and *#2* shown in lanes 3 and 4 and results for Δ*rdr2/3#1* and *#2* shown in lanes 5 and 6. Lanes P1 and P2 show the amplicons corresponding to the non-excised *lox*P-flanked SMG and excised *lox*P-flanked SMG for the *rdr* locus amplified by the corresponding primer pairs. Lane N contains a negative PCR control reaction lacking DNA. The *rdr3* gene was disrupted with *lox*P-*hph*-*lox*P and the *rdr1* and *rdr2* genes were disrupted with *lox*P-*neo*-*lox*P. Primer sequences are shown in Table 1

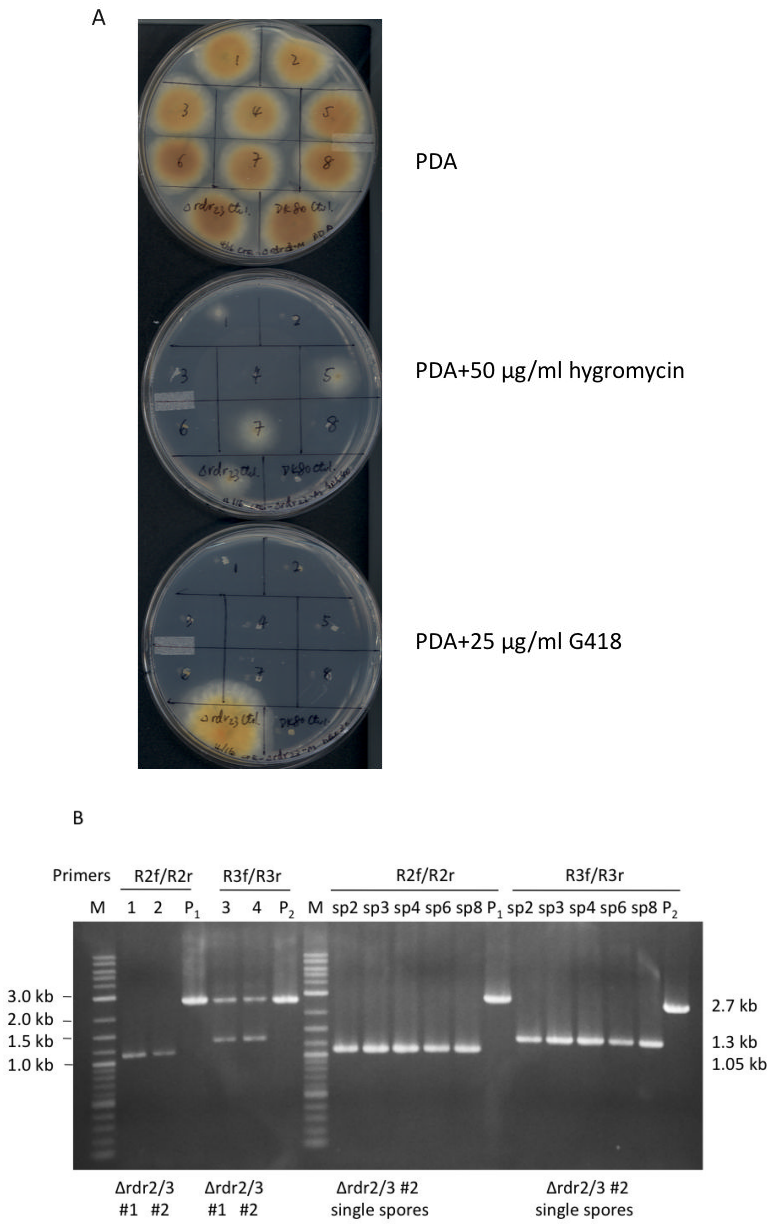

Figure 5.

Antibiotic screening (A) and PCR-agarose gel analysis (B) of single spore-isolates derived from anastomosis-mediated excision recipient strain Δ*rdr2/3#2*. (A). Single condial isolates derived from the recovered Δ*rdr2/3#2* recipient strain after anastomosis with the Cre- donor strain were screened for loss of antibiotic resistance indicating excision of the *lox*P-*hph*-*lox*P SMG from the *rdr3* locus and the *lox*P-*neo*-*loxP* SMG from the *rdr2* locus. Condial isolates were plated on PDA (top plate), PDA containing 50 μg/ml of hygromycin (middle plate) or PDA containing 25 μg/ml G418 (bottom plate). Each set of plates also included strain DK80 (WT) as the SMG-negative control and the untreated SMG-containing Δ*rdr2/3* mutant as the SMG-positive control. Isolates SP2, SP3, SP4, SP6 and SP8 were found to have lost both *hph* and *neo* resistance. (B). As in Figure 4, the DNA ladder is shown in lanes marked M, with marker sizes indicated at the left, and primer pairs are indicated at the top. Lanes P1 and P2 show the 2.7 kb amplicons corresponding to the non-excised *neo*-SMG-containing

rdr1 locus and the non-excised *hph*-SMG-containing *rdr3* locus, respectively. Strain identities are indicated at the bottom. Lanes 1, 2, 3, and 4 show the PCR results for the two recovered recipient strains Δ*rdr2/3#1* and *#2* prior to single spore isolation. PCR analysis of the single condial isolates derived from the recovered Δ*rdr2/3#2* recipient strain SP2, SP3, SP4, SP6 and SP8 described in A confirmed excision of both the *lox*P-flanked *neo* SMG (1.05 kb band) from the *rdr2* locus and the *lox*P-flanked *hgh* SMG (1.3 kb band) from the *rdr3* locus. This contrasts with the presence of amplicons for both the excised (1.3 kb band) and non-excised (2.7 kb band) *hph*-SMGs at the Δ*rdr3* locus observed for the original Δ*rdr2/3#1* amd *#2* recipient strains prior to single condia isolation shown in lanes 3 and 4.

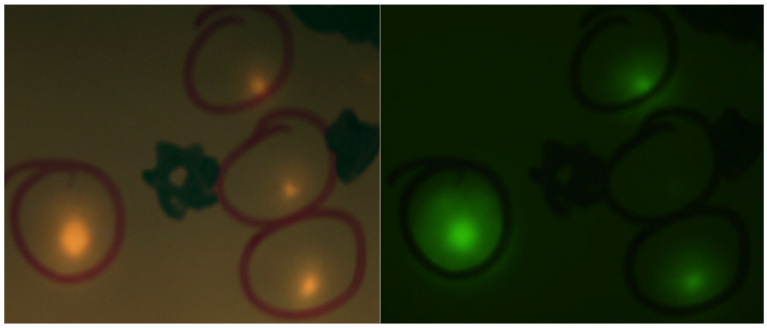

Figure 6.
Fluorescence microscopic screening of condial isolates from *M. robertsii* cre-recipient strains that contained the *lox*P-GFP/BAR-*lox*P cassette. Colonies formed on PDA by condia isolated from a recovered recipient *M. robeertsii* strain containing the *lox*P-GFP/BAR-*lox*P cassette following anastomosis with the *M. robertsii* cre-expressing donor strain were examined for fluorescence under an Olympus fluorescence stereomicroscope model MVX10 (Olympus America Inc., Center Valley, PA). Corresponding bright field and florescence field views are shown in the left and right panels, respectively. Note the colony in the middle right portion of the fluorescent field view that lacks GFP fluorescence, indicating excision of the *lox*P-flanked GFP/BAR cassette during anastomosis. Fluorescence micrographs were taken with an Olympus microscope digital camera DP70 and the software Olympus DP manager version 2.2.1.195 (Olympus America Inc., Center Valley, PA).

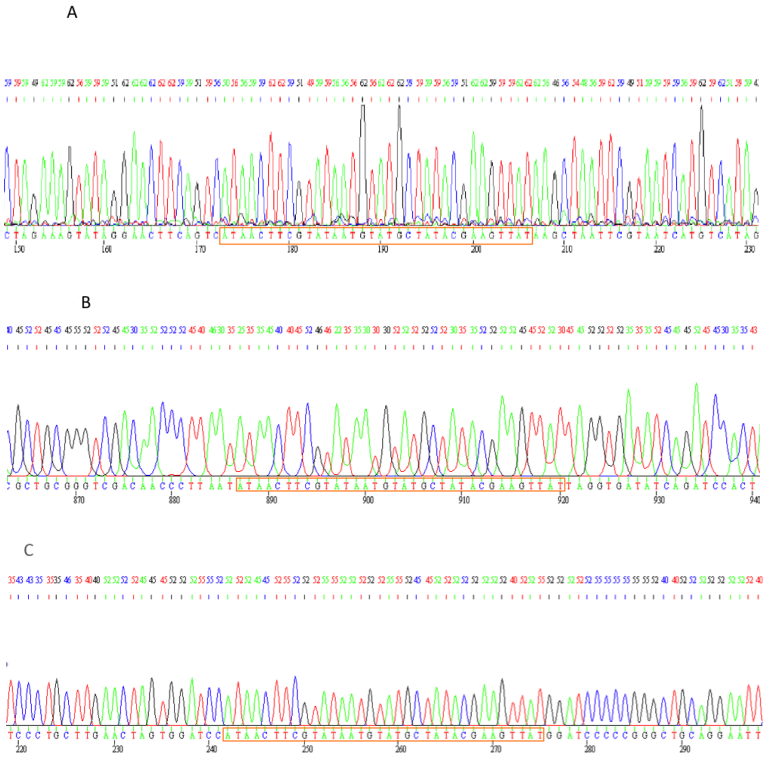

Figure 7.

Sequence analysis of the excision site for the amplicons generated from the excised *M. robertsii lox*P-GFP/BAR-*lox*P element (A), the excised *lox*P-*neo*-*lox*P element at the *C. parasitica rdr2* locus (B) and the excised *lox*P-*hph*-*lox*P element at the *C. parasitica rdr3* locus (C). In each case, the boxed region shows the unique 34 bp *loxP* sequence remaining after excision of the *loxP*-SMG-loxP cassette within the sequence context of the original insertion site.

Table 1

Primers used in this study

Primer	Sequence (5′-3′)	Note
R3nestd1	ACTCTCTCTACTTCTGCATTCTGCGTGCAC	*rdr3* disruption (5′ flanking region)
R3u1	CTGCAGGAATTCGATATCAAGGTAGATATC CAAGGACTCAGCAGG	
R3d2	CCTGCTGAGTCCTTGGATATCTACCTTGATATC GAATTCCTGCAG	*rdr3* disruption (first part of *loxP*-Pro$_{tubB}$-hph-*lox*P)
hphsplit(u)	TAGCTGCGCCGATGGTTTCTACAAAGAT	
hphsplit(d)	ACGCACTGACGGTGTCGTCCATCACAGTT	*rdr3* disruption (second part of *loxP*-Pro$_{tubB}$-hph-*lox*P)
R3u2	CTCATCTTCAGCTCCCTGCTTGAACTAGTGGATCCATAACTTCGT	
R3d3	ACGAAGTTATGGATCCACTAGTTCAAGCAGGGAGCTGAAGATGAG	*rdr3* disruption (3′ flanking region)
R3nestu3	GATATGATGAGGTGGCTGGGCTGTCTTG	
R1nestd1	GTAGACCACTGTAAATGCCAATAGTGC	*rdr1* disruption fragment 1 (5′ flanking region)
R1u1	TGCAGCGTACGAAGCTTCAGCAAGACTGTTGCGAGACCAGGTTAGAGA	
R1d2	TCTCTAACCTGGTCTCGCAACAGTCTTGCTGAAGCTTCGTACGCTGCA	*rdr1* disruption fragment 2 (*loxP*-Pro$_{gpd}$-*neo*-*loxP*)
R1u2	TCTCTTGCTGCTGCTGCTGCTGTAGGCCACTAGTGGATCTGATATCA	
R1d3	TGATATCAGATCCACTAGTGGCCTACAGCAGCAGCAGCAGCAGCAAGAGA	*rdr1* disruption fragment 3 (3′ flanking region)
R1nestu3	TCCGAGTGCATCTCTACACAATCGTCA	
R2nestd1	CTCACGAAAGCTATTCCGTCTGTGCCA	*rdr2* disruption fragment 1 (5′ flanking region)
R2u1	TGCAGCGTACGAAGCTTCAGCTCCCAAGGTCAGCTTCCACTTTCA	
R2d2	TGAAAGTGGAAGCTGACCTTGGGAGCTGAAGCTTCGTACGCTGCA	*rdr2* disruption fragment 2 (*loxP*-pro$_{gpd}$-*neo*-*loxP*)
R2u2	TCGTCCAACTCGTCGCTTTCATCATAGGCCACTAGTGGATCTGATA	
R2d3	TATCAGATCCACTAGTGGCCTATGATGAAAGCGACGAGTTGGACGA	*rdr2* disruption fragment 3 (3′flanking region)
R2nestu3	TACGATTGGCGATGATATCGACGGTTCGT	
Xbalneo(d)	CATTCTAGACACCATGTTGGATAGGGCGAAT	cassette *loxP*-Pro*gpd*-*neo*-*loxP* cloning
Xholneo(u)	ACTCTCGAGGCCAGCAGTAGACACTTGGAAT	
R1f	TTCGAGAATTACACTCAGTCAAATGCTC	check *rdrl* disruption and SMG excision
R1r	TCAATCATCCTCTTGCTGGCT	
R2f	ACATACACACATCTTGCTGCTGCG	check *rdr2* disruption and SMG excision
R2r	TCAATCGTCCTCGTCCAACTC	
R3f	CTTCTGCAATCTTGAGCAGGCTTGCA	check *rdr3* disruption and SMG excision
R3r	TTAGTGCTCATCACGTACGCCAGTA	

Primer	Sequence (5'-3')	Note
NotIcre(d)	TTAGCGGCCGCATGTCCAATTTACTGACCGT	pCPX-cre cloning
SphIcre(u)	ACTGCATGCCTAATCGCCATCTTCCAGC	pCPX-cre cloning; check *cre* integration in *C. parasitica*
Pro-gpd(d)	ACATCGCGTCAGATTCAACCCTCAA	*cre* integration check
Cre SmaI f	TACCCGGGATGTCCAATTTACTGACCGTACACC	pBARGPE-CRE cloning
Cre EcoRIr	ATAGAATTCCTAATCGCCATCTTCCAGCAGGC	
Cre(f)	CCTGTTTCACTATCCAGGTTACGG	check*cre* integration in *M. robertsii*
Cre(r)	CCAATTTACTGACCGTACACCAA	
GFPloxF	CCGGCTCGTATGTTGTGTGGAATTGTG	Check *lox*P-GFP/BAR-*lox*P cassette integration or excision in *M. robertsii*
GFPloxR	ACCCAACTTAATCGCCTTGCAGCAC	