Development of polymorphic microsatellite markers for the genetic characterization of *Knoxdavesia proteae* (Ascomycota: Microascales) using ISSR-PCR and pyrosequencing

Abridged title: Microsatellite development for *Knoxdavesia proteae*

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Abstract

Knoxdaviesia proteae is one of the first native ophiostomatoid fungi discovered in South Africa, where it consistently occurs in the infructescences of the iconic Cape Biome plant, *Protea repens*. Although numerous studies have been undertaken to better understand the ecology of *K. proteae*, many questions remain to be answered, particularly given its unique niche and association with arthropods for dispersal. We describe the development and distribution of microsatellite markers in *K. proteae* through Interspersed Simple Seqeunce Repeat-Polymerase Chain Reaction (ISSR-PCR) enrichment and pyrosequencing. A large proportion of the 31492 sequences obtained from sequencing the enriched genomic DNA were characterized by microsatellites consisting of short tandem repeats and di- and tri-nucleotide motifs. Seventeen percent of these microsatellites contained flanking regions

sufficient for primer design. Twenty-three primer pairs were tested, of which 13 amplified and 12 generated polymorphic fragments in *K. proteae*. Half of these could be transferred to the sister species, *K. capensis*. The developed markers will be used to investigate the reproductive strategy, genetic diversity and dispersal strategies of *K.proteae*.

Keywords: ISSR-PCR, *Knoxdaviesia*, Microsatellites, Ophiostomatoid, Pyrosequencing

Introduction

Knoxdaviesia proteae M.J. Wingf., P.S. van Wyk & Marasas resides in a polyphyletic assemblage (Spatafora & Blackwell 1994) known as the ophiostomatoid fungi (Wingfield *et al.* 1999), which consistently inhabits the fruiting structures (infructescences) of *Protea* species. All evidence suggests that this biogeographically interesting fungus is confined to the single host species, *Protea repens* L. (Roets *et al.* 2009b), where it appears as ascomatal masses on the flowers (Wingfield *et al.* 1988). In addition to *K. proteae*, eight other species belonging to this genus have been described. Of these, *K. capensis* M.J. Wingf. & P.S. van Wyk and *K. wingfieldii* (Roets & Dreyer) Z.W. de Beer & M.J. Wingf. are also known from *Protea* species (Crous *et al.* 2012; Wingfield & Van Wyk 1993), while *K. serotectus* (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. and *K. ubusi* (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. were isolated from declining *Euphorbia ingens* (E. Meyer) Boissier trees in South Africa (Van der Linde *et al.* 2012). *Knoxdaviesia scolytodis* (M. Kolařík) Z.W. de Beer & M.J. Wingf. and *K. cecropiae* (M. Kolařík) Z.W. de Beer & M.J. Wingf. colonise weevil galleries on *Cecropia angustifolia* Trécul trees in Costa Rica (Kolařík & Hulcr 2009; Van der Linde *et al.* 2012). *Knoxdaviesia suidafrikana* (Morgan-Jones & R.C. Sinclair) Z.W. de Beer & M.J. Wingf. and *K. undulatistipes* (Pinnoi) Z.W. de Beer & M.J. Wingf. were recently transferred to *Knoxdaviesia* from *Custingophora* (De Beer *et al.* 2013a).

The association of *K. proteae* with *P. repens* represents an intricate symbiosis in which mites act as the primary fungal dispersers between flower heads and beetles act as the vehicles for mite dispersal (2009a; Roets *et al.* 2007; 2011b). Apart from its modes of dispersal, virtually nothing is known regarding the ecology of *K. proteae*. It is presumed to be a saprobe, but early occupation of infructescences suggests that it may also be able to colonize living tissues (Roets *et al.* 2005; F. Roets, personal observation). This, coupled with

its high level of host specificity, suggests a more complex ecological involvement with *P. repens*.

Although a basic understanding of the ophiostomatoid fungi in *Protea* infructescences has emerged in recent years (2009a; Roets *et al.* 2005; 2011a; 2007; 2011b), very little is known regarding the population biology of these fungi, their reproductive strategies or how these contribute to issues such as dispersal and host association. For example, *P. repens* is a re-seeder in which the infructescences open to release the seeds when their water supply is interrupted, such as after fire (Rebelo 1995). Since both plants and fungi die in these often vast fires, it remains a mystery where the ophiostomatoid fungi encountered in new *P. repens* infructescences come from. The aim of this study was, therefore to develop microsatellite markers as a tool to study the genetic diversity and dispersal biology of *K. proteae*, serving as model for other taxa within this unique ecosystem.

Materials and Methods

Fungal cultures and identification

Strains of *K. proteae* and other *Knoxdaviesia* species were sourced from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Additional *K. proteae* strains were isolated from *P. repens* infructescences from four different locations in the Western Cape Province, South Africa. Isolations were made on Malt Extract Agar (MEA; Merck, Wadeville, South Africa) supplemented with 0.04 g/L Streptomycin Sulfate Salt (Sigma-Aldrich, Steinham, Germany) as described by Roets *et al*. (2006). Individual strains were isolated by sub-culturing a hyphal tip from Water Agar (15 g agar/L) to fresh MEA.

Fungal isolates were grown on MEA overlaid with sterile 3.5 x 3.5 cm^2 cellophane sheets (Product no. Z377597, Sigma-Aldrich, Steinham, Germany). Mycelium was scraped from the cellophane, placed in Eppendorf tubes and shaken vigorously with a vortex mixer in TES buffer (Möller *et al.* 1992), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and glass beads. Subsequent extraction and purification steps followed those of Möller *et al*. (1992). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) was used to determine the quality and quantity of the extracted DNA. The species identity of isolates were confirmed by sequencing the ribosomal

RNA Internal Transcribed Spacer (ITS) regions and performing BLAST (Basic Local Alignment Search Tool) searches on the NCBI nucleotide data base (www.ncbi.nlm.nih.gov). Amplification and sequencing of the ITS region was done with KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA) and followed previously described methods and protocols (White *et al.* 1990).

Microsatellite enrichment

Knoxdaviesia proteae isolates used for microsatellite development included the ex-type strain (CMW 738) collected in the Stellenbosch area and isolate CMW 1043 from the Mossel Bay area (both in the Western Cape Province, South Africa). The ISSR-PCR (Interspersed Simple Sequence Repeat-Polymerase Chain Reaction) technique (Hantula *et al.* 1996; Zietkiewicz *et al.* 1994) was used to establish a pool of microsatellite-enriched genomic DNA. ISSR primers ISSR1 - 5'-DDB$(CCA)_5$, ISSR2 - 5'-DHB$(CGA)_5$, ISSR3 - 5'-YHY$(GT)_5$G, ISSR4 - 5′-HVH$(GTG)_5$, ISSR5 - 5′-NDB$(CA)_7$C, ISSR6 - 5'-NDV$(CT)_8$, and ISSR7 - 5'-HBDB$(GACA)_4$ were used following previously published methods (Santana *et al.* 2009). These primers were applied in 41 different combinations ranging from one to four primers per reaction and amplified using the Fast Start High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). The 50 µl reactions consisted of 5 µl 10x buffer, 1.8 mM $MgCl_2$, 2 µl Dimethyl sulfoxide, 200 µM of each dNTP, 0.1 µM of each primer, approximately 100 ng genomic DNA and 2.5 units of the FastStart High Fidelity Enzyme Blend. Reaction conditions were: 5 minutes at 95°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 45°C and 2 minutes at 72°C, with a final extension step for 7 minutes at 72°C.

The 41 PCR reactions were pooled and purified using the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, Massachusetts, USA) and eluted in low TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California) were used to, respectively, qualify and quantify the microsatellite-enriched DNA in the sample. The final product was sent for pyrosequencing on the 454 Life Sciences/Roche GS-FLX sequencer (Roche Applied Science, Penzburg, Germany) (Margulies *et al.* 2005) at Inqaba Biotechnological Industries, Pretoria, South Africa.

Microsatellite marker development

Duplicate sequences were removed from the 454 data using cdhit_454 (identity threshold = 0.98) (Niu *et al.* 2010). Microsatellites were identified with MSATFINDER ONLINE 2.0 (Thurston & Field 2005) using the REGEX search engine and default search parameters to search for perfect repeats. Data were analyzed using Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA, USA).

Primers were designed with the help of MSATFINDER and PRIMER3PLUS (Untergasser *et al.* 2007) and sequence analysis was done with BIOEDIT 7.1.3.0 (Hall 1999). To ensure that the identified tri-nucleotide loci were not located in coding regions, the relevant pyrosequencing reads were subjected to a BLAST search using the least conservative engine, BLASTtx (www.ncbi.nlm.nih.gov). Polymorphism was assessed by amplifying and sequencing all loci in a set of 10 *K. proteae* isolates. This set consisted of the ex-type strain, CMW 738, five *K. proteae* strains isolated from Gouritz, two from Outeniqua Pass, and one each from Uniondale and Sir Lowry's Pass, all in the Western Cape. To test amplification of the loci in other *Knoxdaviesia* species, three *K. capensis* (CMW 974, CMW 997 and CMW 11962), two *K. cecropiae* (CMW 22991 and CMW 22993), one *K. scolytodis* (CMW 22995), three *K. serotexi* (CMW 34100, CMW 36767 and CMW 36768) and two *K. ubusi* (CMW 36769 and CMW 36770) isolates were used.

To test random association of loci, the data from the polymorphism tests were used to determine two different measures of assessing pair-wise linkage disequilibrium. Fisher's exact test was calculated with GENEPOP version 4.2 (Rousset 2008) and $\bar{r}_d$, the index of multilocus linkage disequilibrium, was calculated with MULTILOCUS version 1.3b (Agapow & Burt 2001). False discovery rate using GENPOP was avoided as recommended by Benjamini & Yekutieli (2001).

Results and Discussion

Roche 454 sequence data

A total of 31492 sequences with an average read length of 281.2 base pairs were generated using pyrosequencing. Of these, 16820 (53 %) were unique and 5408 (17 %) had sufficient sequence data flanking the microsatellite loci for primer design. Although contigs were not constructed from our 454 data, the proportion of amplifiable sequences was slightly higher

than the 14 % noted by Santana *et al*. (2009), who used the same Roche 454 technology. High-throughput sequencing of an enriched pool of microsatellites, therefore, provided an immense improvement on the traditional practice of cloning and Sanger sequencing (Santana *et al.* 2009). The procedure was not only less laborious and time-consuming, but also afforded the advantage of being able to choose loci of interest.

Identification of microsatellites

Since microsatellites with higher-than-average repeat numbers are more likely to be polymorphic (Dettman & Taylor 2004; Dutech *et al.* 2007; Goldstein & Clark 1995), only those with repeat numbers greater than five were tested. Twenty three primer pairs were initially designed and tested. Of these, 13 loci amplified in *K. proteae* and 12 generated polymorphic fragments (Table 1). The other was a compound microsatellite, $(ACAG)_{6-7}(C)_n$, and was discarded as its mutational mechanism may differ significantly from that of perfect repeats. The number of alleles detected ranged from 2 to 9 with a mean of 4.75 ± 0.55 – similar to the average allele number of 5.4 ± 0.4 reported for fungi (Dutech *et al.* 2007). Neither Fisher's exact test nor the $\bar{r}_d$ value indicated significant pairwise linkage after performing the linkage disequilibrium tests.

Cross-species transferability

In the three *K. capensis* isolates, six of the 12 microsatellite markers amplified in isolates CMW 997 and CMW 19962 and four in CMW 974. All six markers were polymorphic between *K. proteae* and *K. capensis* and polymorphism could also be detected within *K. capensis* for loci Ti-4, Ti-6 and T5. The cross-species transferability rate was higher than expected based on previous fungal studies. For example, in a survey of fungal studies Dutech *et al*. (2007) reported a transferability rate of approximately 34 %. However, this high rate of marker transferability was not surprising, as *K. capensis* is phylogenetically most closely related to *K. proteae*, and these two species group separately from other *Knoxdaviesia* species in phylogenetic trees (De Beer *et al.* 2013b; Wingfield *et al.* 1999).

Distribution of microsatellites in Knoxdaviesia proteae *454 sequence data*

Microsatellite distribution was described based on the results of the Multipass search implemented in MSatFinder Online version 2.0 (Thurston & Field 2005). Similar results were generated using the other MSatFinder engines, but Multipass allowed detection of

Table 1 Polymorphic microsatellites developed for *Knoxdaviesia proteae*

Locus	Motif	Genbank accession	Primer names	Primer sequences (5'-3')	Fluorescent tag	Size range	Alleles		Transferable
							Gouritz[a]	Total[b]	
D2	$(tc)_{16}$	KF924611	KX1-1	GAGACATACTGGACTGTACACATTCAT	VIC	107-115	4	9	-
			KX1-2	CGTCCTGTAGTGGCTATCCTG	-				
Ti-2	$(ctt)_{16}$	KF924612	KX2-1	TGTCGGGTCCTGTGTAACTG	PET	108-144	4	8	-
			KX2-2	CGGATTGTATCAGTTGTCCTCA	-				
Ti-3	$(ctt)_{13}$	KF924613	KX3-1	CGGGACTCCACTCTCTCAAG	VIC	173-200	1	3	-
			KX3-2	CCGGACTCTAGACGTTGAGG	-				
Ti-4	$(gtc)_{12}$	KF924614	KX4-1	AAGAAAAGCCAAGGGGAGAG	NED	166-184	2	5	*K. capensis*[c]
			KX4-2	ACTTACAGGCCAGGACCACA	-				
Ti-5	$(cag)_{11}$	KF924615	KX5-1	ACGACAGCAGATGCATGAAG	NED	115-127	4	5	-
			KX5-2	GCTGTCTTGCTGCTGAACTG	-				
Ti-6	$(acc)_{9}$	KF924616	KX6-1	CAAAATGGTGCCCTATGACC	6-FAM	189-198	3	4	*K. capensis*[c]
			KX6-2	ACAAGCCAGAGTTTGGAGGA	-				

Ti-7	$(ctt)_9$	KF924617	KX7-1	AGACGCTTCCATCTCGTTTC	6-FAM	96-108	2	3	*K. capensis*
			KX7-2	GGAGATGGCGAGAGAAGTTG	-				
T2	$(atgg)_7$	KF924618	KX8-1	GACGACGATCACAGGACGAC	6-FAM	104-116	2	4	*K. capensis*
			KX8-2	CAAGCCCTCAAAGTTGCTTC	-				
T3	$(agtg)_8$	KF924619	KX9-1	TGAGATTGCGAGTGTGCTTC	VIC	100-132	2	4	-
			KX9-2	GGTGTGCATCAATTGTTCGT	-				
T4	$(attc)_6$	KF924620	KX10-1	CCCCATCAGTTCACTGACATC	PET	183-195	4	5	*K. capensis*
			KX10-2	GACCACAGTTGGGAAAATCG	-				
T5	$(agtg)_7$	KF924621	KX11-1	AGAGAGCCTTCCCAAAAGGT	6-FAM	185-189	2	2	*K. capensis*[c]
			KX11-2	GCGAAAGGGAACATAAATCG	-				
T6	$(ctgt)_{16}$	KF924622	KX12-1	GGGCAGAACCGTTCATATTC	VIC	183-231	4	5	-
			KX12-2	AACCCTGCGTCAGACACC	-				

[a] Alleles from the 5 strains isolated from the Gouritz area

[b] Alleles from all 10 strains used to test polymorphism

[c] Polymorphic in *K. capensis*

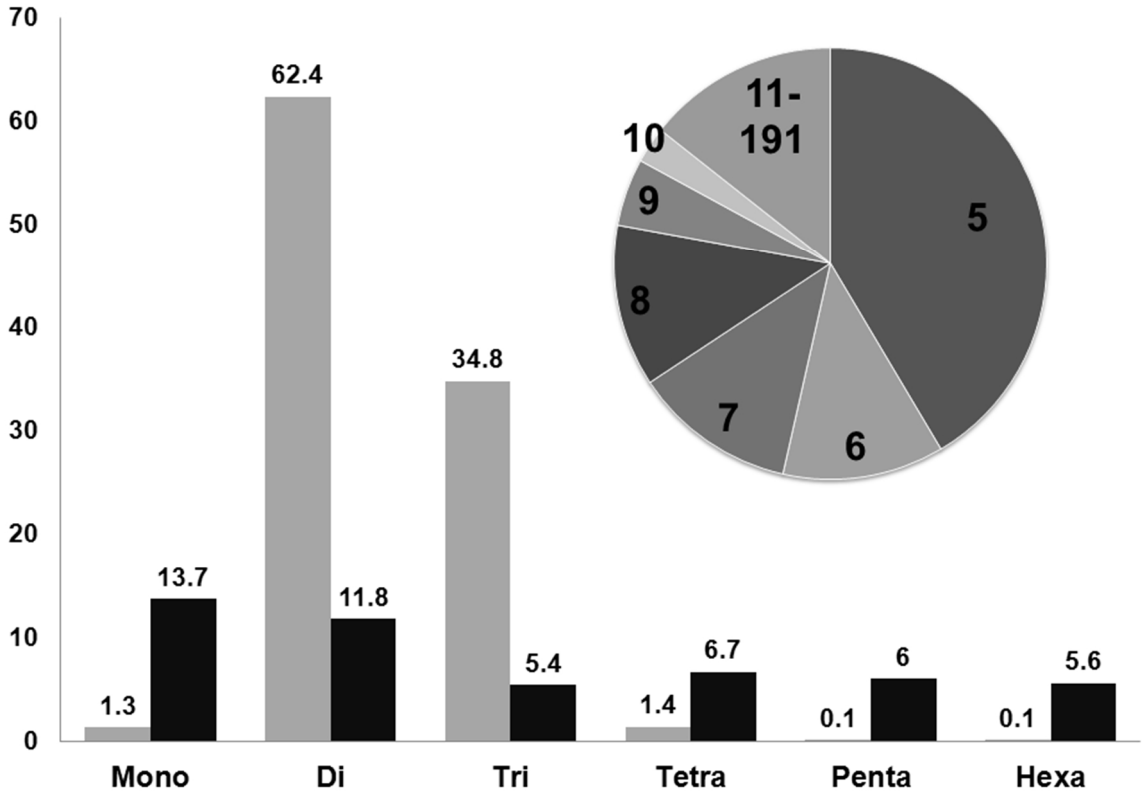

Fig. 1 Distribution of microsatellite classes and tandem repeat units in the 454 data. The histogram indicates the percentage occurrence (*grey bars*) and the mean number of tandem repeats (*black bars*) observed for each microsatellite class. Standard errors are too small to be displayed on the graph. The pie chart displays the prevalence (in %) of the different tandem repeat numbers (shown on chart). Tandem repeat numbers 6, 7 and 8 are in equal proportion and 11 to 191 are merged

additional microsatellite loci in all classes except the mono-nucleotides. For these analyses, duplicate sequences were excluded at a cdhit_454 threshold of 0.95.

Although the microsatellite enrichment strategy employed in this study might have skewed the observed distribution of these motifs in *K. proteae*, our results are consistent with those for fungi. For example, studies considering whole-genome sequences (Karaoglu *et al.* 2005; Katti *et al.* 2001; Lim *et al.* 2004) have shown that fungal microsatellites tend to be short and that mono-, di- and tri-nucleotide motifs predominate. Di- and tri-nucleotides were encountered frequently in our data, while mono-nucleotide motifs comprised only 1.3 % of the identified microsatellites (Fig. 1). Such frequencies have also been reported following analysis of an ISSR-PCR enriched library for *Fusarium circinatum* Nirenberg & O'Donnell

(Santana *et al.* 2009), suggesting that the relatively few mono-nucleotide microsatellites observed in this study might be a consequence of the specific enrichment method used.

Although tandem repeat numbers of up to 191 were observed, short repeats were most common (Fig. 1). More than 40 % of microsatellites consisted of 5 tandem repeats and 86.7 % had 5-10 repeats. This is roughly the same as reported by Lim *et al*. (2004), who found that 90 % of their dataset was comprised of microsatellites with 5-7 repeat units. As expected and observed in previous studies (Karaoglu *et al.* 2005; Santana *et al.* 2009), the average tandem repeat number of the microsatellites decreased as the motif complexity increased. This may be due to lower mutation rate and therefore lower polymorphism in long motifs (Chakraborty *et al.* 1997; Kruglyak *et al.* 1998), as well as a bias towards contraction mutations in large microsatellite alleles (Xu *et al.* 2000).

Conclusions

This study is the first to develop microsatellite markers for a species of *Knoxdaviesia* and also the first to apply microsatellite enrichment coupled with pyrosequencing to ophiostomatoid fungi. The 12 polymorphic markers developed here will be applied to investigate the genetic diversity and dispersal of this arthropod-vectored fungus within the Cape Floristic Region of South Africa. Half of the markers also have the potential to be used to study the closely related *K. capensis*, establishing an opportunity for comparison of these ecologically similar fungi.

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