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Chris Martine  
*Bucknell University, ctm015@bucknell.edu*

Ally Boni  
*Bucknell University*

Elizabeth Capaldi  
*Bucknell University, elizabeth.capaldi@bucknell.edu*

Gemma Lionheart  
*Bucknell University*

Ingrid E. Jordon-Thaden  
*University of California - Berkeley*

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Evidence of rock kangaroo seed dispersal via faecal seed storage in a tropical monsoon community

Christopher T. Martine¹, Alexandra J. Boni¹, Elizabeth A. Capaldi¹, Gemma E. Lionheart¹,² and Ingrid E. Jordon-Thaden¹,³

¹Department of Biological Science, Bucknell University, Lewisburg, Pennsylvania, USA
Email: christopher.martine@bucknell.edu
²Department of Biology, University of Mississippi, University, Mississippi, USA
³University and Jepson Herbaria, University of California, Berkeley, California, USA

Abstract

While some of the plant species of the ‘Sandstone Country’ along the escarpment of western Arnhem Land produce fleshy fruits and appear to rely on biotic methods of seed dispersal, little is known about the methods by which this is achieved – and few potential dispersers co-occur in the sandstone outcrop communities. For the present study, scat collections were made on outcrops in the northeastern area of Kakadu National Park with the hope of uncovering relationships between local frugivores and fruit-producers, and providing evidence for seasonal storage of mammal-dispersed seeds in scat prior to germination. The goals of the present project were to collect and identify sandstone community macropod scat, determine the identity of seeds present in the scat, and provide support for the role of browser/grazer macropods as effective seed dispersers via faecal seed storage in an otherwise disperser-poor local fauna. Scat containing seeds was identified as belonging to the Black Wallaroo (Macropus bernardus), a rare and locally-endemic macropod considered an intermediate browser/grazer. These seeds were successfully germinated and the seedlings identified using molecular phylogenetic techniques as Gardenia fucata (Rubiaceae), an endemic rock-specialist species – thus establishing the first confirmation of effective seed dispersal by a ‘rock kangaroo’ in this region and the first identification of a seed disperser for this uncommon Gardenia species. The results provide support for the role of browser/grazer macropods as occasional effective seed dispersers of rock-specialist plant species in the northern monsoon tropics of Australia via faecal seed storage.

Introduction

Effective seed dispersal is defined not only by the movement of seeds, but also by the successful recruitment of new individuals following that movement (see Schupp et al. 2010). For fleshy-fruited plant species relying on endozoochory, identifying effective dispersal requires knowledge of whether a given animal ingests the seeds, whether those seeds survive passage through the digestive tract, and whether seed germination follows deposition of seeds in faecal material.
Production of fleshy fruits is relatively common among the woody rock-specialist plants occurring in the monsoonal and fire-prone ‘Sandstone Country’ along the escarpment of western Arnhem Land (Northern Territory, Australia). While numerous species appear to rely on endozoochorus seed dispersal, little is known about the methods by which this is achieved in this habitat – and few potential dispersers co-occur with the fruit-producing plants found growing there (Menkhorst & Knight 2011).

Rock-dwelling macropods are fairly common in the northern monsoon tropics of Australia and reflect an unusually high regional species diversity there (Telfer & Bowman 2006). The Nabarlek (*Petrogale concinna*), Short-eared Rock Wallaby (*Petrogale brachyotis*), Common Wallaroo (*Macropus robustus*) and Black Wallaroo (*Macropus bernardus*) (Fig. 1) all occur in sympatry (Menkhorst & Knight 2011). Because these northern ‘rock kangaroo’ taxa are largely nocturnal and generally shy, empirical knowledge of their behaviour, diet and distribution is limited (Richardson 2012).

In a 1998 paper, Telfer et al. compiled Aboriginal knowledge of rock kangaroo feeding habits that included numerous anecdotal accounts of opportunistic macropod frugivory – even though the dominant understanding of foraging behavior has defined these animals as intermediate browsers/grazers (Tuft et al. 2011). A later study (Telfer and Bowman 2006) using scat contents to explore niche separation among northern macropods found that at least one of the species, *Macropus bernardus*, may be able to inhabit the most rugged and severe habitat because of its ability to utilise leaves, fruits and seeds from a range of rock-specialist plants during dry seasons. In conjunction with this work, Telfer et al. (2006) designed a scat identification key to determine current

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**Fig. 1.** Black Wallaroo (*Macropus bernardus*). (Stephen Zozaya, by permission)
distribution patterns for macropod species in the Top End region of the Northern Territory; a study that set the stage for a follow-up analysis correlating these distributions with habitat characteristics (Telfer et al. 2008).

Coupling the work of Telfer and colleagues with field observations, Martine & Anderson (2007) postulated that rock-dwelling macropods play an important role in short-distance seed dispersal of sandstone endemic plants through a three-step process consisting of fruit ingestion, “faecal seed storage,” and “seasonal redispersal” via wet season rains. However, the authors did not test experimentally whether particular animal taxa actually functioned in this role for specific plant taxa.

The goals of the present project were to:
1. Collect and identify sandstone community macropod scat, examine it for seeds and, if present, germinate them; and
2. Identify the resultant plant(s) species using morphology and, if needed, molecular tools.

**Materials and Methods**

In May 2013, 80 scat pellets were collected from upper elevation scat piles on sandstone outcrops in the vicinity of Merl Campground and Cahills Crossing, East Alligator region, Kakadu National Park (Northern Territory, Australia). Two investigators gathered scats using a haphazard sampling scheme during which all intact scats encountered were collected during a single day over a period of roughly eight hours. At the time of collection, the scats were run through the identification key published by Telfer et al. (1996). Although the scats were relatively dry at collection, they were allowed to further air dry before being packed in paper coin envelopes and shipped to Bucknell University for future work.

In July 2013, the scats were dissected and searched for the presence of seeds. Seeds were removed and directly sown into a soil tray without pre-treatment in order to ensure that any germination success might be based on natural processes alone (notably the passage through an animal’s gut and, perhaps, time passed in the scat). Because seedling recruitment on rock outcrops appears to be highest in cracks and fissures where scats/seeds are covered with accumulated detritus (CTM, pers. obs.), experimental seeds were shallowly planted below the soil surface. The tray was then placed in a growth chamber with temperature, light, and humidity settings previously found to be successful for two species of *Solanum* endemic to the area where the scats were collected (as per Lionheart 2014). The soil was kept moist at all times.

Once seedlings were apparent, they were transplanted into pots and moved to the Bucknell University research greenhouse, where a temperature and light regime matching present conditions in Kakadu was already in place. Following establishment, leaf material was removed and dried on silica for future DNA work.
In April 2014, CTM returned to Kakadu, compared leaves of the germinated greenhouse plants to plants on the site where the scat was collected and, finding a potential match, collected voucher material. This material was examined and identified by staff at the Northern Territory Herbarium in Palmerston, then accessioned at the Manning Herbarium at Bucknell University for later use in DNA extractions.

Dried leaf material was extracted using a modified CTAB protocol (Doyle & Doyle 1987). Leaf tissue was pulsed using a GenoGrinder (SPEX Sample Prep) and steel beads in 2 ml microfuge tubes. The CTAB plant solution was incubated for 30 min at 37°C, centrifuged, and moved to a clean tube. The aqueous solution was extracted with chloroform: isoamyl alcohol (24:1) twice. The DNA was precipitated with ice cold 100% isopropanol for 20 min at -20°C, then pelleted for 20 min at 4°C in the centrifuge at 7000 rpm. The DNA pellets were cleaned with two consecutive washes of ethanol, 75% and 95%, respectively, and re-suspended in 100 ml ddH₂O. The re-suspended pellet was incubated at 37°C with RNase for one hour, and let to sit overnight at 4°C. The DNA was then frozen at -20°C for storage.

The ITS (internal transcribed spacer) gene region was amplified using PCR with the following protocol for a 30 μl reaction volume: 2 min denaturing at 95°C; 29 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at 56.8°C, and elongation for 30 sec at 72°C; with a final extension for 5 min at 72°C. The reaction mixture for the PCR includes 6 μL GoTaq Flexi buffer (Promega, Madison, WI), 2 μl of MgCl₂ (1 mM), 1 μl each of forward and reverse primers (0.2 mM, Invitrogen, Carlsbad, CA), 0.6 μl dNTP mix (0.2 mM, Promega), and 1.25 units of GoTaq Flexi (Promega). The primers designed for these studies were as used in Jordon-Thaden et al. (2010) for the ITS of the ribosomal DNA sequence. The primers amplified the ITS2, ITS1, and the 5.8 S rDNA region. This was done with the ITS-18 forward 5’-GCA TGT TTT CCC AGT CAC GAC GGA AGG AGA AGT CGT AAC AAG G-3’ which includes an M13 extension (the last 19 bases). The reverse ITS-25 primer was 5’-ACT TCA GGA AAC AGC TAT GAC GGG TAA TCC CGC CTG ACC TGG-3’ which also includes an M13 extension (the first 21 bases). For Sanger sequencing of the ITS region, the M13 extension primer alone was used for the PCR products that had been generated with the M13 extension attached to the ITS forward and reverse primers. The M13 extension for forward primer is 5’-GCA TGT TTT CCC AGT CAC GAC-3’ and reverse is 5’-ACT TCA GGA AAC AGC TAT GAC-3’. The PCR products were cleaned with the Promega Wizard cleaning system (Promega), and Sanger sequenced with an ABI sequencer at the Heck Genomics Institute at Pennsylvania State.

Sequenced gene regions were processed with Geneious R7 (Biomatters Ltd.) and then compared via BLAST search to confirm the match and the identification of the taxon grown from seeds recovered from the scat.

Finding a generic match, multiple ITS accessions of congeners were downloaded, along with an outgroup taxon, and used for phylogenetic comparisons with the two
“unknown” accessions. Sequences were aligned in Geneious R7 (Biomatters Ltd.) and a maximum likelihood ITS tree was generated with GARLI (Zwickl et al. 2006), with bootstrap values based on 10,000 reps. Novel sequences generated from the individual collected from scat and the specimen taken from the potential match in the field were deposited in GenBank (see ID numbers in tree figure).

Results

Use of the Telfer et al. (2006) scat key confirmed that the scat collected on our site was that of the Black Wallaroo, an escarpment-restricted species listed as Near Threatened by the IUCN (Woinarski 2008) largely because of a small total global population limited to a geographic range of about 30,000 km$^2$ (Telfer & Calaby 2008). Of the 80 scats examined, only one contained seeds, with seven seeds removed from the same sample. These seeds germinated at a rate of 100% without pre-treatment.

Plant voucher material collected at the scat collection site was identified by Ian Cowie at the Northern Territory Herbarium as Gardenia fucata R.Br. ex Benth. (Rubiaceae), a small tree endemic to the sandstone escarpment country (Puttock 1997) in the Top End region of the Northern Territory (Fig. 2). While no previous ITS accession existed in GenBank for G. fucata (or any other Australian Gardenia), the BLAST search confirmed that the ITS sequences of the greenhouse-grown seedlings (GenBank KP657895) and the wild-collected specimen of G. fucata (GenBank KP657896) were closely allied to other members of the genus Gardenia. Using Gardenia ITS sequences from GenBank (and Ixora pavetta as the outgroup taxon) for comparison to our greenhouse-grown and wild-collected accessions, phylogenetic analysis provided support for their close alliance (Fig. 4). With this evidence, we can conclude that the seed found in the scat was most likely Gardenia fucata, a species occurring in fairly low abundance in the site where the scats were collected.

Discussion

While previous scat analyses (Telfer & Bowman 2006) and compilation of local Indigenous knowledge (Telfer et al. 1998) suggested that Black Wallaroos occasionally eat fruits and seeds, no empirical evidence for their role as effective seed dispersers has been previously established. Our results show that Black Wallaroos in the East Alligator region of Kakadu National Park occasionally ingest the fruits of Gardenia fucata and that those seeds are not only passed intact in scat but are also able to germinate at a high rate (100%; n=7). In
the sandstone habitats where this rock-specialist plant species occurs, few other potential seed dispersers are present – meaning that even if Black Wallaroos (and potentially other rock macropods) are not frequent frugivores, bouts of frugivory and seed dispersal may still play an important role in recruitment of new individuals of Gardenia fucata. The fruits of G. fucata begin ripening in July (peak dry season) and are not especially fleshy, the mesocarp instead having a fibrous nature (Puttock 1997). Local Indigenous knowledge compiled by Telfer & Garde (2006) includes accounts of M. bernardus (and two other rock kangaroo species) consuming the leaves of G. fucata, but not the fruits.

**Outcrop interactions** The sandstone outcrops of the escarpment country offer a unique suite of underlying opportunities and challenges to their resident flora and fauna. Because outcrops typically retain water, they offer a refuge for plant communities requiring more available resources than the surrounding eucalypt savannah communities (Brock 2001) and thus may support a richer suite of invertebrate and vertebrate animals. Outcrops support communities that are distinct from the surrounding landscape – including numerous endemic species (Brennan 1986; Brock 2001). At the same time, these outcrop communities, while similar to the nearby plateau country, also remain distinct from it by virtue of their smaller size and island-like isolation. In the most pronounced cases they serve as refugia for drought-intolerant species that, formerly more widespread, were forced to retreat to these islands of tolerable habitat during the Pleistocene – the best example perhaps being Allosyncarpia ternata, a tree common in previously widespread rainforests that is now found only in refugial monsoonal gorges along the Arnhem Plateau escarpment (Russell-Smith 2009).

These restricted rock outcrops may have also served (and continue to serve) as refugia for Macropus bernardus. Telfer & Bowman (2006) suggest that M. bernardus, the species with smallest range (Fig. 3) of six Macropus species in Australia (Woinarski 2008; Coulson and Eldridge 2010), was forced to retreat to these habitats during glacial periods of the Pleistocene and subsequently adapted to exploit rock-specialist plants (many of them chemically-defended). All rock kangaroos are considered refuge-dependent, with disjunct occurrences across broad geographic regions reflecting the scattered distribution of compatible habitat (Tuft et al. 2011); and this is strongly pronounced in M. bernardus. An analysis of environmental correlates found that M. bernardus distributions, more so than the patterns for three sympatric rock-dwelling macropods, are especially linked to the presence of rugged, rocky terrain (Telfer et al. 2008).

For plant communities on outcrops, these habitats operate like terrestrial islands, with isolated populations for which gene exchange is challenged through the limited movement of pollinators and seed dispersers (Martine & Anderson 2007; Roche et al. 2014). While little has been done on the effects of outcrop-to-outcrop distance on gene exchange, the relationship between two sandstone specialist sister species, Solanum asymmetriphyllum and S. sejunctum, shows that the taxa remain distinct (Brennan et al. 2006; Martine et al. 2006) even though they can hybridise and are separated by just 10 km at the nearest point in their combined distribution (Gilman et al. 2014). Previous inferences (Anderson
Martine et al. (2016) have noted that, although long-distance pollinators are rare visitors to flowers on outcrops, most of the services are provided by small-bodied bees flying over short distances.

Although we can assume that gene migration is also limited by seed dispersal challenges (Symon 1979; Martine & Anderson 2007), little has been done to show it. Martine & Anderson (2007) theorised that rock wallabies were key players in the movement of outcrop seeds, but recognised that this was likely to occur within outcrops rather than between them. Because these macropods are poorly adapted for movement across flat ground (a consequence of being exceptionally adapted for climbing around rocks) (Telfer & Bowman 2006), they are most likely to facilitate short distance dispersal within outcrops. This might be accomplished through nightly dry season foraging bouts where seeds are moved into ‘faecal seed storage’ in/around daytime roosting spots (Telfer & Griffiths 2006), followed by ‘seasonal redispersal’ via wet season downpours – an inference supported by the frequent occurrence of young plants in steep washes, ravines and deep cracks (CTM, pers. obs.).

The present study shows that rock-dwelling macropods may occasionally function as effective seed dispersers for at least one sandstone outcrop endemic and, by nature of the locations and manner in which seeds were collected, appear to provide faecal seed storage as per Martine & Anderson (2007). Occasional seed and fruit opportunism on the part of the macropods (see Jarman 1994; Telfer & Bowman 2006) may thus be as important to the plants as to the animals, particularly during seasons when the availability of grasses and foliage is reduced.

Whether this interaction then leads to seasonal redispersal during the monsoon season is left to be determined, but knowing that seeds stored in faeces on upper elevation rocks can germinate without any additional treatment aside from the presence of a moist substrate allows us to assume that seedling recruitment can follow redistribution of seeds by water and gravity. Telfer et al. (2006) found that although scats of the Short-tailed Rock Wallaby may persist in nature for over two years, most scats are lost within six months, ostensibly being degraded and washed away by wet season rains. Faecal seeds redispersed during these rains might then reap the benefits of increased access to nutrients and carbon supplied by the faeces (Kobayashi et al. 2011).

**Fig. 3.** Map of the Top End region of the Northern Territory showing approximate range of *Macropus bernardus* (red) based on Menkorst & Knight (2011) and locations (dots) of *Gardenia fucata* collections accessioned at the Northern Territory Herbarium (from I. Cowie). (Map drawn with ESRI ArcGIS)
Given the low numbers of seeds recovered in this study, we cannot discount the potential for post-dispersal seed predation to render our findings moot. Ants and rodents are known to predate seeds released in scat (e.g. Hulme 1998; Manzano et al. 2010; Peco et al. 2014); and both are in abundance in the study area. However, seeds moved but not consumed during bouts of post-dispersal predation (see Azacarate & Manzano 2011) may benefit from redispersal similar to that hypothesised above.

There is growing evidence that small- and medium-sized mammals throughout the northern monsoon tropics of Australia have recently experienced and continue to face major declines, even in Kakadu National Park and other protected areas considered to be ecologically intact (Woinarski et al. 2001; Ziembicki et al. 2012; Woinarski & Fisher 2013). Further work is needed to help understand and protect the links between these mammals and the plants that may depend on them, at least in part, for their long-term survival. Descriptive studies like this one provide individually small steps that should prove cumulatively integral to conservation efforts, particularly for organisms whose natural histories are as poorly understood as those highlighted here.

Conclusions

This study establishes the potential role of rock macropods as dispersers of rock-specialist plants in the monsoon tropics of Australia. Scat containing seeds was identified as belonging to the Black Wallaroo, a rare and locally-endemic macropod considered an intermediate browser/grazer. Seeds were successfully germinated and seedlings were identified using molecular techniques as Gardenia fucata, an endemic rock-specialist species – thus establishing the first confirmation of effective seed dispersal by a rock kangaroo in this region and the first identification of a seed disperser for this uncommon Gardenia species. The results provide support for the role of browser/grazer macropods as occasional effective seed dispersers of rock-specialist plant species via faecal seed storage.

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