Low temperature and low moisture storage of seeds of rare and threatened taxa in the endemic Western Australian genus *Dryandra* (R. Br.) (Proteaceae)

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ABSTRACT

Seeds from 23 collections of 13 threatened taxa in the Western Australian endemic genus *Dryandra* (R. Br.) (Proteaceae) (*Dryandra acanthopoda, D. anatona, D. fuscobractea, D. ionthocarpa, D. longifolia* subsp. *calcicola, D. mimica, D. mucronulata* subsp. *retrorsa, D. nivea* subsp. *uliginosa, D. seneciifolia, D. serra, D. serratuloides* subsp. *perissa, D. squarrosa* subsp. *argillacea, D. viscida*) were stored for one year at -20°C and at moisture contents of 5 ± 1%. Overall there was no difference in germination response pre- or post-storage (85 ± 3% vs. 80 ± 4%), although there was substantial inter- and intra-specific variation in both per cent germination and rate of germination for collections within the genus. Per cent germination ranged from 52–100% pre-storage and 24–100% post-storage. Time to 50% germination varied from 14 to 350 days pre-storage, with post-storage 18 to 197 days. In 14 collections no difference in ability to germinate was detected after the one-year period of storage; however, five collections of *Dryandra longifolia* subsp. *calcicola, D. mucronulata* subsp. *retrorsa*, one collection of *D. seneciifolia* and two collections of *D. ionthocarpa* showed a decline in germination ability after storage. The entire collection of *Dryandra viscida*, one collection of *D. nivea* subsp. *uliginosa* and the remaining two collections of *D. ionthocarpa* increased in per cent germination after storage. Using predictions from a seed viability equation, these preliminary results indicate that *ex situ* seed storage under low moisture and low temperature conditions is possible as a means of long-term maintenance of seed of threatened *Dryandra* species.

INTRODUCTION

The genus *Dryandra* (R. Br.) (family Proteaceae, sub-family Grevilleoideae, tribe Banksieae, sub-tribe Banksiinae) is a prominent endemic genus of Western Australia, with 93 species and 34 infraspecific taxa known from the South West Botanical Province (George 1999). It is the third largest genus in the family Proteaceae in Western Australia, and the majority of species are found on laterite soils in and around the agricultural wheatbelt, or on deep sands on the southern coast or northern sandplains, and are an important component of many vegetation types in the South West. All species are woody perennials ranging in size from prostrate shrubs to small trees. Most have seed enclosed by woody follicles that are released on heating. They are closely related to the genus *Banksia*, differing in the presence of a prominent involucre and in the shape and arrangement of the seed follicles (Sainsbury 1985). Many species are useful for the cut flower trade (e.g. *D. formosa*) and as food for native bird species.

This important genus of southwestern Australian ecosystems is being threatened by broadscale clearing for agriculture, habitat fragmentation, weed invasion, and rising salinity. Most taxa are also considered highly susceptible to the soil-borne root fungus *Phytophthora cinnamomi* (Malajczuk and Glenn 1981; Rockel *et al.* 1982; Shivas 1989; Wills 1992). And many have a restricted range and are endangered or critically endangered (Brown *et al.* 1998). The Western Australian Department of Conservation and Land Management has listed 74 *Dryandra* taxa as 'declared rare or priority flora' considered to be under threat (Atkins 1998). Nine of these are Declared Rare Flora and two of these nine are considered Critically Endangered under International Conservation Union (IUCN) criteria (Brown *et al.* 1998). Many of these taxa are facing extinction within the next 10 years.

Loss of populations and substantial reduction in population size may not necessarily lead to immediate species extinction, but inevitably results in loss of genetic

diversity. Genetic variation enables plants to adapt to changing environmental and ecological conditions, as well as providing resistance to pests and disease, and is critical for the long term survival of most species. *Ex situ* programs incorporating genebanks, or long-term storage facilities for genetic material, can be used as an interim solution to prevent this loss of genetic diversity, or used as a last resort in preventing the extinction of the species. One of the most cost-effective methods for genebanking in plants is the long-term storage (i.e. a minimum of 50 years) of seeds at low (-20°C) temperatures (Roberts 1989).

Seeds from native plants in southwestern Australia are predominantly orthodox in nature, having the potential to be stored successfully at reduced moisture contents and temperatures for long periods of time (Morse *et al.* 1993). The highly compact nature of seeds make them ideal for storage and low temperature storage is more economical than maintaining collections of living plants in botanic gardens. Despite the usefulness of seed storage as a conservation measure, there is limited information available on seed storage behaviour of Australian species. Refer to Ewart 1908 and 1925 for information on room temperature storage; Duyker 1964, Boland *et al.* 1980, Turnbull and Martensz 1982, Omran *et al.* 1989, Cochrane and Kelly 1996 and Cochrane and Monks 1998 for information on low temperature storage; and Touchell and Dixon 1993 and 1994 for information on cryostorage.

The aim of this study was to investigate the ability of seeds of the Western Australian endemic genus *Dryandra* to survive desiccation to 5 ± 1% moisture content and storage at low temperature (-20°C). From this study it should be possible to use an existing viability equation framework to extrapolate the effectiveness of these conditions for long-term storage of this genera and many other Western Australian plant species. The data will aid the off-site management and conservation of critically endangered and other conservation flora.

MATERIALS AND METHODS

Study taxa

The taxa selected for study (Table 1) represent a broad range of taxonomic and ecological variation found within the genus *Dryandra*, ranging from *D. serratuloides* subsp. *perissa*, known from 200 km north of Perth on laterite soils in mallee woodlands, to *D. longifolia* subsp. *calcicola*, located some 800 km east of Perth in coastal limestone heath near Esperance (Fig. 1). All species are conservation taxa, either gazetted as Declared Rare Flora or listed as Poorly Known Taxa, and in need of further survey (Priority Flora) under the Conservation Codes for Western Australia (Atkins 1998).

Seed collection

Seeds were obtained from wild populations between January 1993 and December 1997, and the site locations have been abbreviated to protect the confidentiality of conservation flora populations. Collections were made from a minimum of 10 plants per population to ensure the capture of a broad range of genetic variation (Brown and Briggs 1991). Seed testing and storage occurred at the Department's Threatened Flora Seed Centre (TFSC), a seed-based genebank for the conservation of genetic material of rare and threatened Western Australian taxa.

Seed storage

International standards for long-term storage of orthodox seeds for genetic conservation recommend storage for the long-term at moisture contents of 5 ± 1% (4% for oily seeds, 6% for starchy seeds) in hermetic containers at -18°C or lower (Cromarty *et al.* 1990). Under these standards, seeds should remain viable for hundreds of years. In this study extracted seeds were dried for six to eight weeks in a dehumidifying room at 15°C and 15% relative humidity, or dried over silica gel in small desiccators for up to one month until moisture contents had reduced to 5 ± 1%. Seed moisture content was determined by the low constant temperature oven dry method (ISTA 1996). Dried seeds were hermetically sealed in laminated foil bags and frozen at –20°C. After one year, seeds were thawed, allowed to re-hydrate for 24 hours to prevent imbibition damage, and their ability to germinate was re-tested.

Seed germination

Cochrane and Kelly (1996) demonstrated that seeds of many *Dryandra* species contain no dormancy mechanisms and require no treatment to elicit germination. On this basis, no germination pre-treatment was given to seeds after extraction from woody follicles and surface sterilising with a 10% solution of 40gL^{-1} of sodium hypochlorite for five minutes. Seeds were germinated in 90 mm glass Petri dishes on a 0.75% (w/v) agar solution in incubation cabinets, using a 12 hour photoperiod at a constant 15°C temperature. A 2% solution of *Previcure* fungicide was added to the agar solution to inhibit fungal attack.

A series of germination trials were conducted between 1993 and 1999 and were undertaken on fresh seeds, within one month of collection, and on seeds stored at -20°C for a period of one year. Seed viability was not assessed through artificial means such as chemical or x-ray analysis. An indication of viability through such analyses, although useful to assess the potential health of a seed batch, does not produce a whole plant. The seed resources of conservation taxa are often limited in supply and whole plants, derived from these seed germination trials, were necessarily required for further recovery, research or educational purposes.

Pre- and post-storage germination treatments were identical. Petri dishes were checked twice weekly and germination was determined by radicle emergence. Sample sizes for germination trials ranged from 20 to 75 seeds and were dependent on the number of seeds collected.

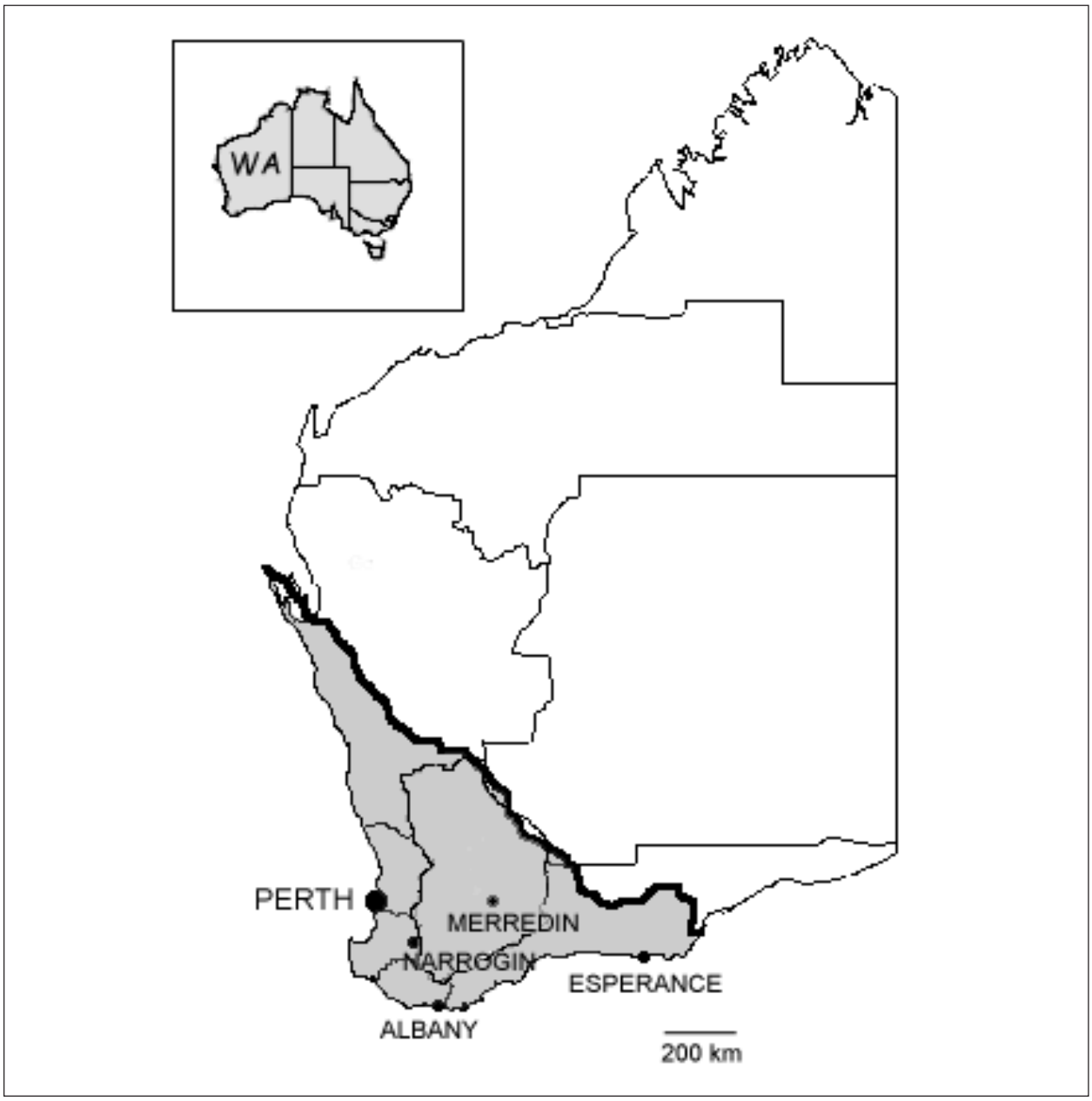

Figure 1. Western Australia and the species-rich South West Botanical Province with Department of Conservation and Land Management regional boundaries delineated. All taxa used in this study were obtained from within the Province indicated by shading. Inset: Australia.

Many of these trials included no replication due to the scarcity of seed (with numbers of seed used in each trial noted in Table 1). Cumulative germination percentages have been calculated on the basis of total seed numbers and germination time has been expressed as: the time in days from sowing to initial germination (T_i), time to 50% germination (T_{50}) time to final germination (T_f), and total time from initial to final emergence of all germinable seeds (T_t). Trials were allowed to run until all seed were either germinated or considered dead (mouldy or soft).

Statistical analysis

Germination results were expressed as a percentage of the number of seeds sown. Pre- and post-storage germination data were compared using a Chi-Square analysis on count data. A 90% confidence interval was used due to low seed numbers. A Student's T-test was used to compare paired means for time to initial germination, time to 50% germination, time to final germination and total germination time. Percentage data was arcsine transformed prior to analysis.

TABLE 1
Percentage germination of *Dryandra* seeds pre- and post-storage. N = number of seeds used. Chi Square analysis ($\chi 2$) shows statistical significance (df=1) *P<0.1, ** P<0.05, *** P<0.01, **** P<0.001

SPECIES	YEAR OF COLLECTION	SITE	PRE-STORAGE GERMINATION %	POST-STORAGE GERMINATION %	N	$\chi 2$
Dryandra acanthopoda A.S.George (ser. Armatae)	1993	CR	90	93	80	ns
Dryandra anatona A.S. George (ser. Ilicinae)	1997	EPT	96	99	150	ns
Dryandra fuscobractea A.S.George (ser. Armatae)	1996	BGR	95	95	40	ns
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1997	KN	52	92	100	****
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1993	KN	60	79	54	*
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1993	KS	60	24	55	***
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1990	KS	100	63	26	*
Dryandra longifolia subsp. *calcicola* A.S. George (ser. Armatae)	1997	TC	93	70	60	*
Dryandra mimica A.S. George (ser. Gymnocephalae)	1997	W	100	100	30	ns
Dryandra mucronulata subsp. *retrorsa* A.S. George (ser. Foliosae)	1997	C	94	76	100	**
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	BMS	70	55	80	ns
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	GBR	92	84	149	ns
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	SR	98	98	100	ns
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1995	TR	91	92	148	ns
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1996	WW	98	94	100	ns
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1994	WW	89	100	71	**
Dryandra seneciifolia R. Br. (ser. Obvallatae)	1993	RGP	83	73	97	**
Dryandra seneciifolia R. Br. (ser. Obvallatae)	1993	HH	92	75	27	ns
Dryandra serra A.S. George (ser. Concinnae)	1993	SS	80	59	20	ns
Dryandra serra A.S. George (ser. Concinnae)	1997	DRNR	70	60	86	ns
Dryandra serratuloides subsp. *perissa* A.S. George (ser. Capitellatae)	1994	MT	94	100	107	ns
Dryandra squarrosa subsp. *argillacea* A.S. George (ser. Armatae)	1994	TR	96	100	100	ns
Dryandra viscida A.S. George (ser. Gymnocephalae)	1993	HH	52	67	135	*

RESULTS

Influence of storage on seed germination

Overall there was no difference in pre- and post-storage germination for the study taxa (Student's t=1.19, df=21, p<0.01). Mean germination for the 23 collections pre-storage was 85 ± 3% (range 52 to 100%), with one year post-storage mean germination of 80 ± 4% (range 24 to 100%). However, there was considerable inter- and intra-specific variation in per cent germination within the genus (Table 2). Pre- and post-storage results for all collections of *D. ionthocarpa,* and *D. mucronulata* subsp. *retrorsa, D. nivea* subsp. *uliginosa* (WW94), *D. longifolia* subsp. *calcicola, D. seneciifolia* (RGP93) and *D. viscida* were different. Germination of *D. longifolia* subsp. *calcicola, D. mucronulata* subsp. *retrorsa, D. seneciifolia* and two collections of *D. ionthocarpa* (KN93 and KN97) declined after post-storage, whilst the remaining four collections increased after storage.

Influence of storage on rate of germination

Although the effect of storage at low moisture and low temperature on germination rate was not consistent across the genus, there was no overall difference between pre- and post-storage T_i, $T_{50,}$ or T_f for the genus (Table 3). For many collections, the onset and completion of germination of fresh seeds was more rapid. In two collections each of both *D. serra* and *D. seneciifolia* pre- and post-storage germination were similar, although germination was consistently slower in post-storage trials (Fig. 2). At day 50, pre-storage per cent germination was 30%, 75%, 86% and 83% for the four collections, compared with post-storage of 20%, 33%, 70%, and 67% respectively. Seed germination in *D. mucronulata* subsp. *retrorsa* also followed this course (Fig. 2). *D. nivea* subsp. *uliginosa* (GBR97 and SR97) showed similar trends for rate of post-storage germination as the above, but the final per cent germination showed little difference (Fig. 3).

Conversely, the onset and completion of germination was more rapid after storage for seeds of *D. acanthopoda*, *D. squarrosa* subsp. *argillaceae*, and *D. ionthocarpa* (KN97) (Fig. 4). Completion of germination post-storage was reached for the three taxa at day 24, 28 and 35 respectively, although on these same days pre-storage germination was 45%, 50%, and 0% respectively.

Seeds of the remaining collections showed very little difference in rate of germination pre- and post-storage (Fig. 5).

DISCUSSION

Intra-specific differences in seed germination and germination time within the 23 collections confirm that seed viability and germinability are not constant over space or time. Environmental and genetic effects may contribute to these differences. Environmental stress during seed maturation, habitat differences and population size and condition can contribute to differences in seed germination between populations of the same species, and between successive years of collection. Genetic differences between and within taxa, and between populations of the same taxa, can also manifest in variation in seed germination. Although the taxa investigated were in the main highly germinable, it is possible that dormancy may have prevented full germination of all seeds within some collections, showing species, site and seasonal specificity.

Influence of storage on seed germination

Observations of differing responses to storage may be due to variation in maturity and vigour of seeds. This variation may be reflected in the reduced lifespan of the seeds under constant storage conditions (Smith 1984). Seeds collected immature may lose viability more rapidly than mature seeds, and the viability of seeds from years when ripening and harvesting conditions were poor may decline more rapidly (Roberts 1972a). Plus mechanical damage from cleaning and drying can also reduce storage life (Roberts 1973). In conjunction with these influences, it is likely that differences in pre- and post-storage germination also represent gains or losses in dormancy and / or viability over time. Dormancy is often most pronounced immediately after seed collection (Ellis *et al.* 1985), and many species from southwestern Australia produce seeds which will not germinate directly after dispersal, despite adequate conditions of light, temperature and moisture (Bell *et al.* 1993 and Bell 1999). Embryos are often immature and require a period of after-ripening to develop the ability to germinate. The achievement of maximum germination in the genus *Dryandra*, whether pre- or post-storage, may require seed to be kept at room temperature for a period of time prior to incubation.

In previous research on germination in *D. viscida* after storage at low moisture and low temperature for 2 years, the application of Gibberellic Acid (GA_3) at 25 mgL^{-1} stimulated seeds to reach 86% within 66 days (compared to 277 days to reach 67% in untreated seeds) (A. Cochrane unpublished data). This suggests that for optimal germination applications of the growth hormone may be required. The use of growth hormones to break dormancy has been used previously in Western Australian species exhibiting low per cent germination under standard conditions of temperature, light and moisture (Bell *et al.* 1993 and 1995; Schatral 1996; Cochrane *et al.* 1999).

Although few taxa in this study displayed dormancy, a major limitation to effective research into storage in native seed is the lack of knowledge of dormancy breaking treatments in the seed germination tests that are used to estimate viability. And although dormancy is considered biologically advantageous (Villiers 1972), it presents a problem for research into the response of seeds to a variety of storage regimes. Alternative methods to determine viability are not always considered reliable (Robert 1972b and Lush 1982), or are prohibitively costly for small genebanks (x-ray machines). In addition, the assessment of seeds' response to storage is only truly effective if the promotion of germination of dormant seeds can be

TABLE 2
Time (days) to initial (T_i), 50% (T_{50}), final (T_f) and total time (T_t) to germination for *Dryandra* seed pre- and post-storage.

SPECIES	YEAR OF COLLECTION	SITE	PRE-STORAGE				POST-STORAGE			
			T_i	T_{50}	T_f	T_t	T_i	T_{50}	T_f	T_t
Dryandra acanthopoda A.S.George (ser. Armatae)	1993	CR	13	28	69	56	13	18	24	11
Dryandra anatona A.S. George (ser. Ilicinae)	1997	EPT	20	23	65	45	22	28	57	35
Dryandra fuscobractea A.S.George (ser. Armatae)	1996	BGR	21	21	41	20	19	19	33	14
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1997	KN	45	350	350	305	24	27	35	11
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1993	KN	34	62	94	60	34	45	119	85
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1993	KS	34	62	171	137	24	-	31	7
Dryandra ionthocarpa A.S. George (ser. Ionthocarpae)	1990	KS	26	32	60	34	32	50	54	22
Dryandra longifolia subsp. *calcicola* A.S. George (ser. Armatae)	1997	TC	18	18	35	17	18	19	25	7
Dryandra mimica A.S. George (ser. Gymnocephalae)	1997	W	20	20	59	39	39	22	67	28
Dryandra mucronulata subsp. *retrorsa* A.S. George (ser. Foliosae)	1997	C	34	41	66	32	40	60	92	52
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	BMS	23	32	23	23	28	54	63	35
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	GBR	29	29	39	10	28	53	95	67
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1997	SR	18	18	25	7	31	31	77	46
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1995	TR	11	14	56	45	16	19	54	38
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1996	WW	17	16	55	38	21	23	63	42
Dryandra nivea subsp. *uliginosa* A.S. George (ser. Niveae)	1994	WW	12	17	26	14	19	18	54	35
Dryandra seneciifolia R. Br. (ser. Obvallatae)	1993	RGP	19	18	47	28	22	33	98	76
Dryandra seneciifolia R. Br. (ser. Obvallatae)	1993	HH	17	21	66	49	26	34	85	59
Dryandra serra A.S. George (ser. Concinnae)	1993	SS	19	23	64	45	38	60	146	108
Dryandra serra A.S. George (ser. Concinnae)	1997	DRNR	36	55	102	66	50	88	145	95
Dryandra serratuloides subsp. *perissa* A.S. George (ser. Capitellatae)	1994	MT	12	15	40	28	11	18	53	42
Dryandra squarrosa subsp. *argillacea* A.S. George (ser. Armatae)	1994	TR	20	17	90	70	14	24	28	14
Dryandra viscida A.S. George (ser. Gymnocephalae)	1993	HH	39	173	400	361	25	197	277	252

TABLE 3
Mean time (days) for initial, final, 50% and total germination pre- and post-storage for 23 accessions (range in brackets) across all *Dryandra* taxa.

	PRE-STORAGE (DAYS)	POST-STORAGE (DAYS)	STUDENTS T=
Mean time to initial germination (T_i)	23 (11-45)	26 (13-50)	ns
Mean time to 50% germination (T_{50})	48 (14-350)	42 (18-197)	ns
Mean time to final germination (T_f)	89 (23-400)	77 (24-277)	ns
Mean total germination time (T_t)	66 (7-361)	51 (7-252)	ns

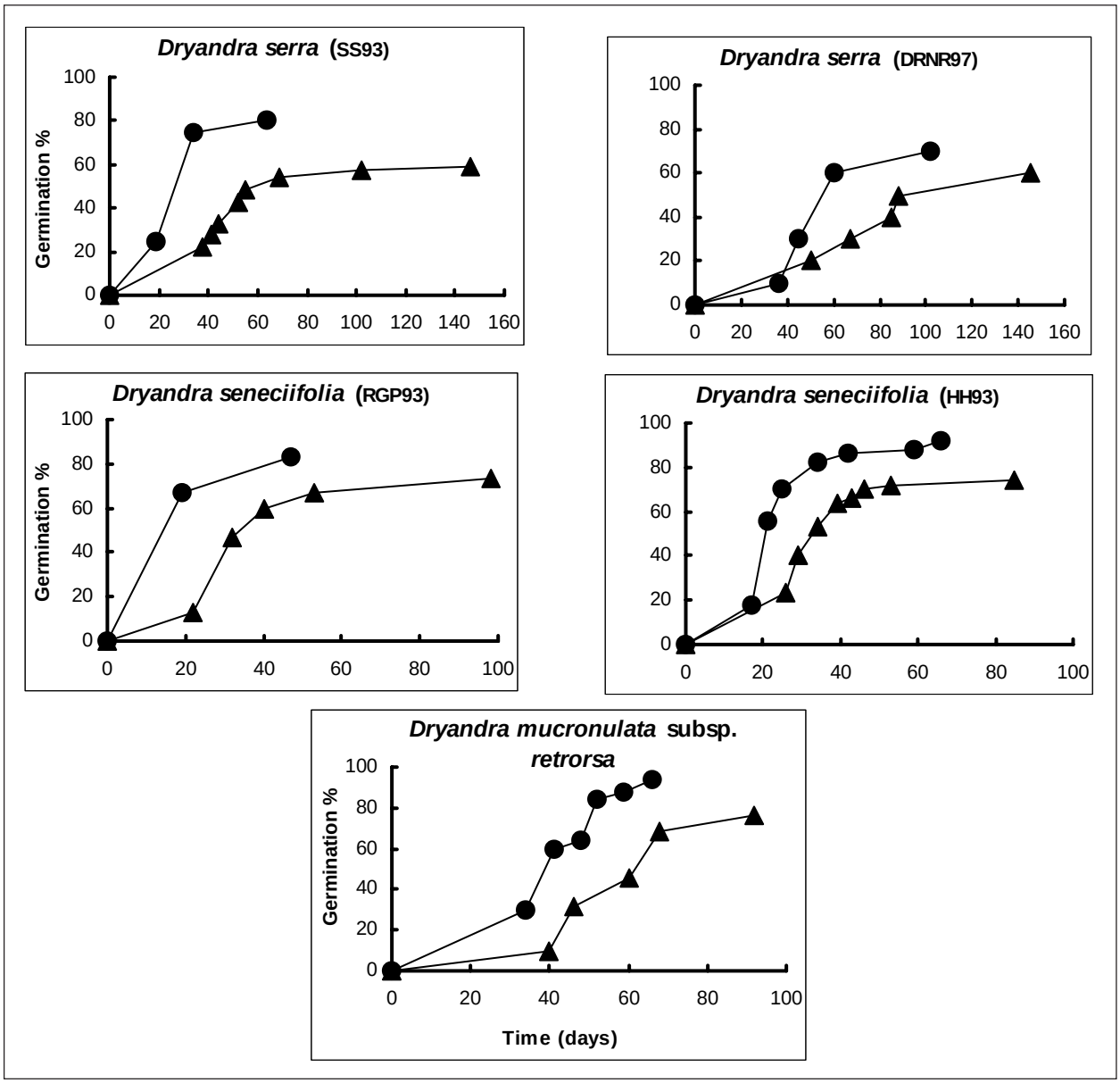

Figure 2. Cumulative germination for five collections demonstrating differences in percentage and rate of germination pre- ● *and post-* ▲ *storage.*

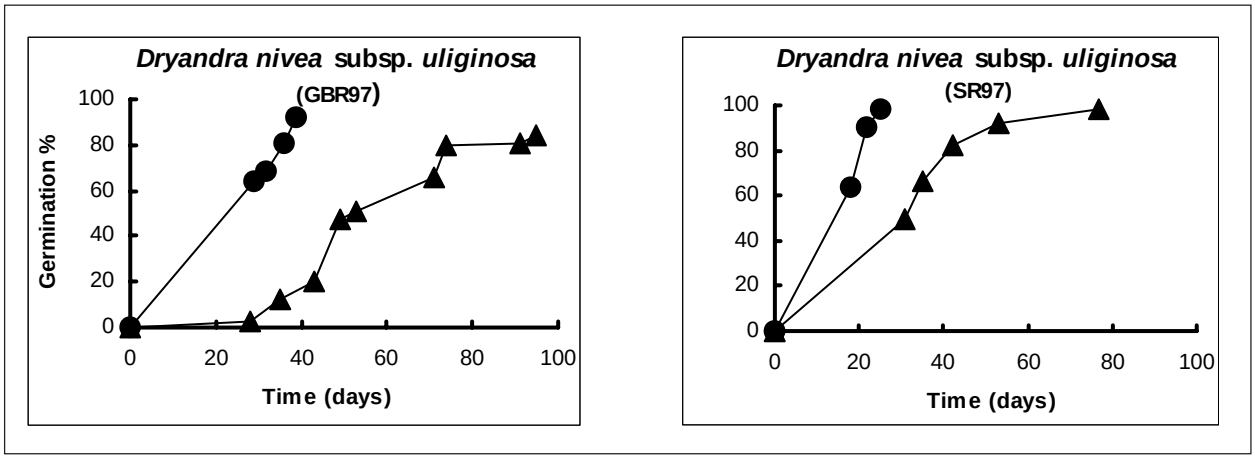

Figure 3. Cumulative germination for two collections demonstrating similarities in percentage germination but differences in rate of germination pre- ● *and post-* ▲ *storage.*

established. Seeds are of little use unless samples can be germinated when required for monitoring or recovery (Morse *et al.* 1993), and continued research into the germination requirements of threatened taxa is advocated.

Influence of storage on rate of germination

Differences in the rate of germination for fresh and stored seeds may also be related to dormancy, viability or vigour of that seed. The actions of drying and / or freezing appear to have rendered the seeds of *D. acanthopoda* and *D. squarrosa* subsp. *argillaceae* more germinable, demonstrated by an increase in the rate of germination post-storage without a corresponding change in per cent germination. On the other hand, an increase in rate of germination post-storage for one collection of *D. ionthocarpa* (KN97) coincided with a significant gain in germinability (52% pre-storage vs. 92% post-storage). In those collections exhibiting a slower rate of germination after storage, it is possible that dormancy or loss of viability has occurred during storage. Dormancy may be overcome, in time, during the germination trial as the trial duration increases. Alternatively, loss of vigour of seeds during storage may be manifested in a slower rate of germination, coinciding with a significantly lower post-storage per cent germination demonstrated by *D. mucronulata* subsp. *retrorsa* and *D. seneciifolia* (RGP93).

Despite the individual variation in pre- and post-storage per cent and rate of germination, this study has demonstrated that storage of seeds of many species of the Western Australian endemic genus *Dryandra* at low moisture and low temperature does not compromise viability in the short term. Extrapolation of results is required if data from short-term research is to provide advice on *ex situ* conservation of the species for the long term. A seed viability equation has been developed that provides a framework for extrapolation of these short term results to the long term, offering predictions compatible with results after 125 years of storage (Ellis and Roberts 1980).

The scientific management of *ex situ* collections, and their use in recovery and reintroduction into managed environments, is of vital importance to the maintenance of genetic diversity and for future flora recovery. The low-cost methods of maintaining genetic diversity undertaken by the Threatened Flora Seed Centre plays an essential complementary role in an integrated conservation strategy that incorporates on-ground management, research, education and recovery for a wide range of threatened taxa.

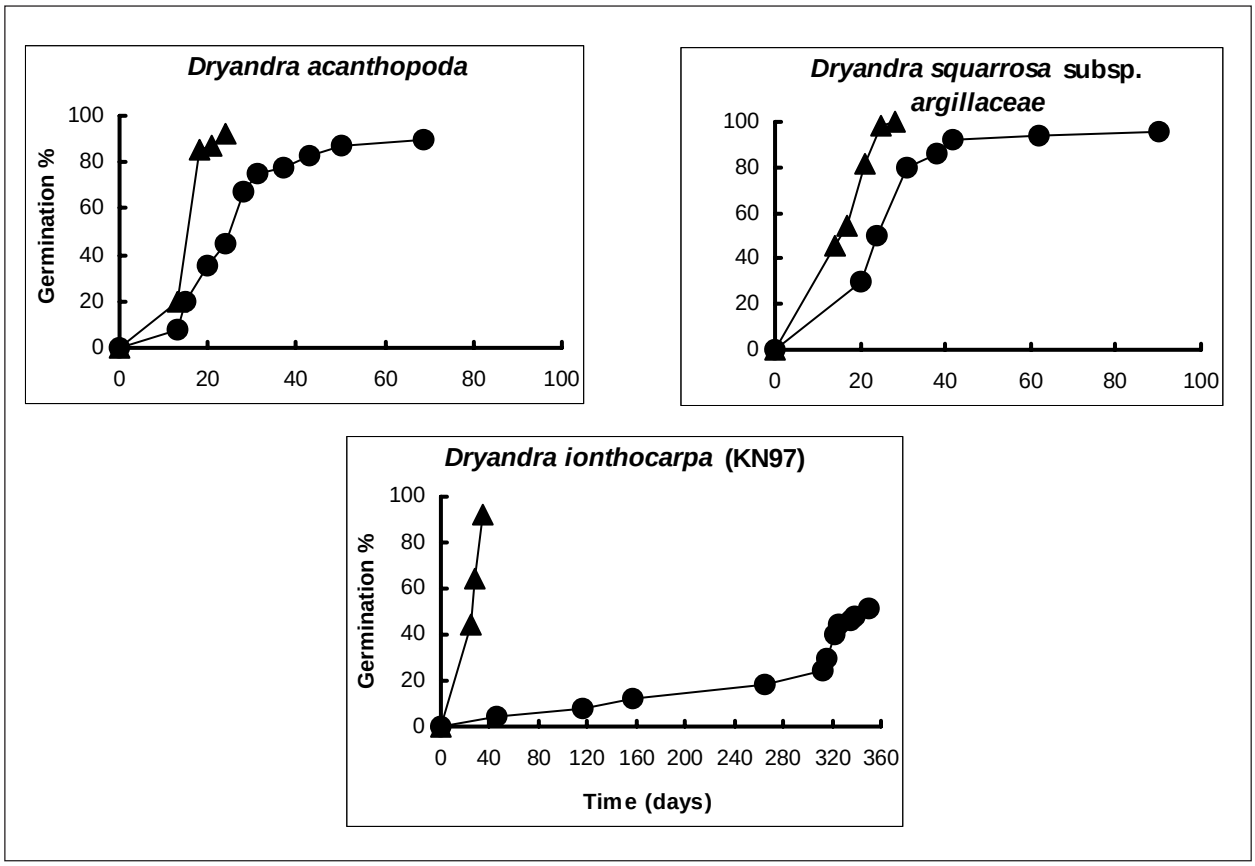

Figure 4. Cumulative germination for three collections demonstrating differences in rate of germination pre- ● *and post-* ▲ *storage.*

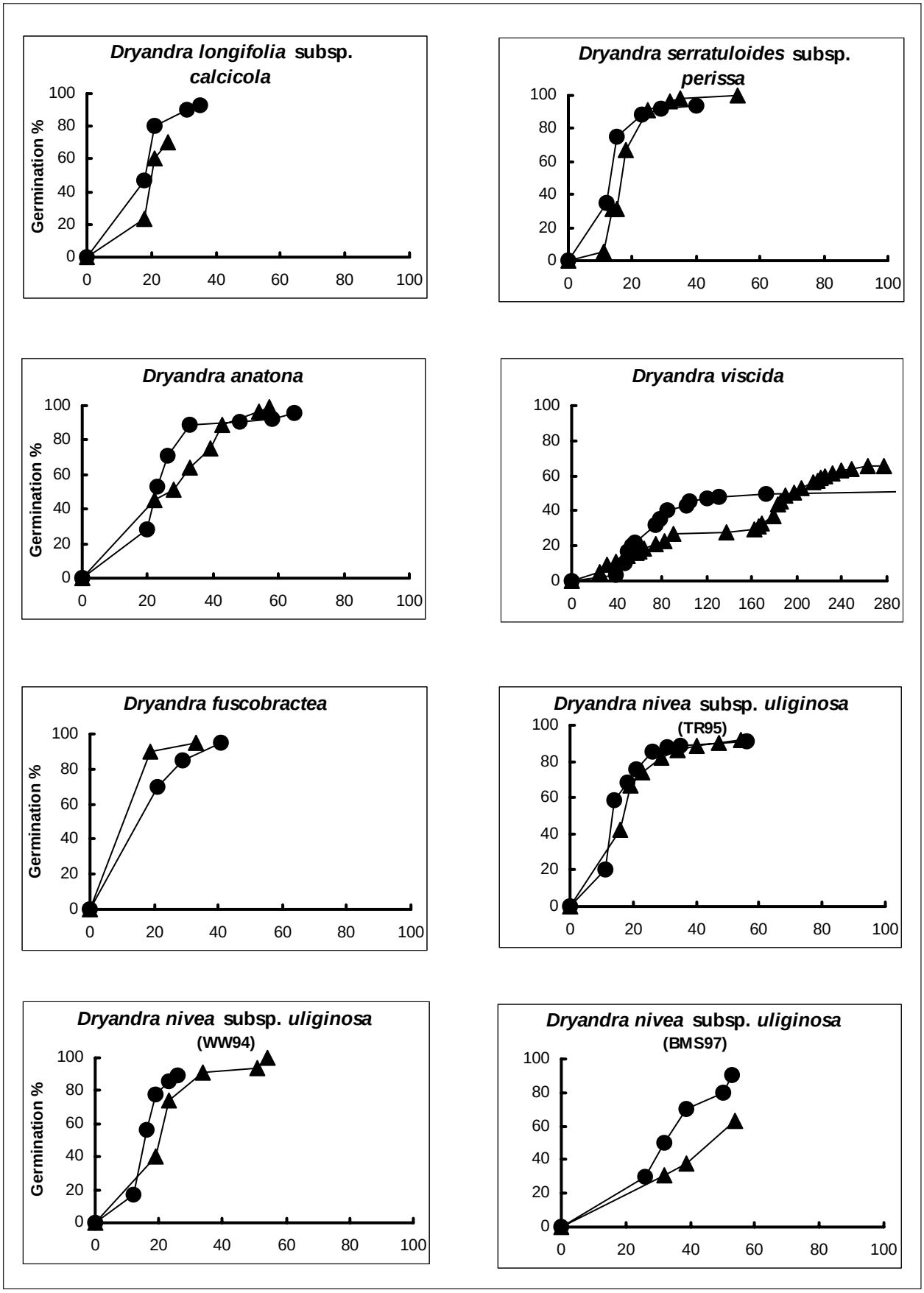

Figure 5. Cumulative germination for 13 collections demonstrating similarities in rate of germination pre-● and post-▲ storage (continued overleaf).

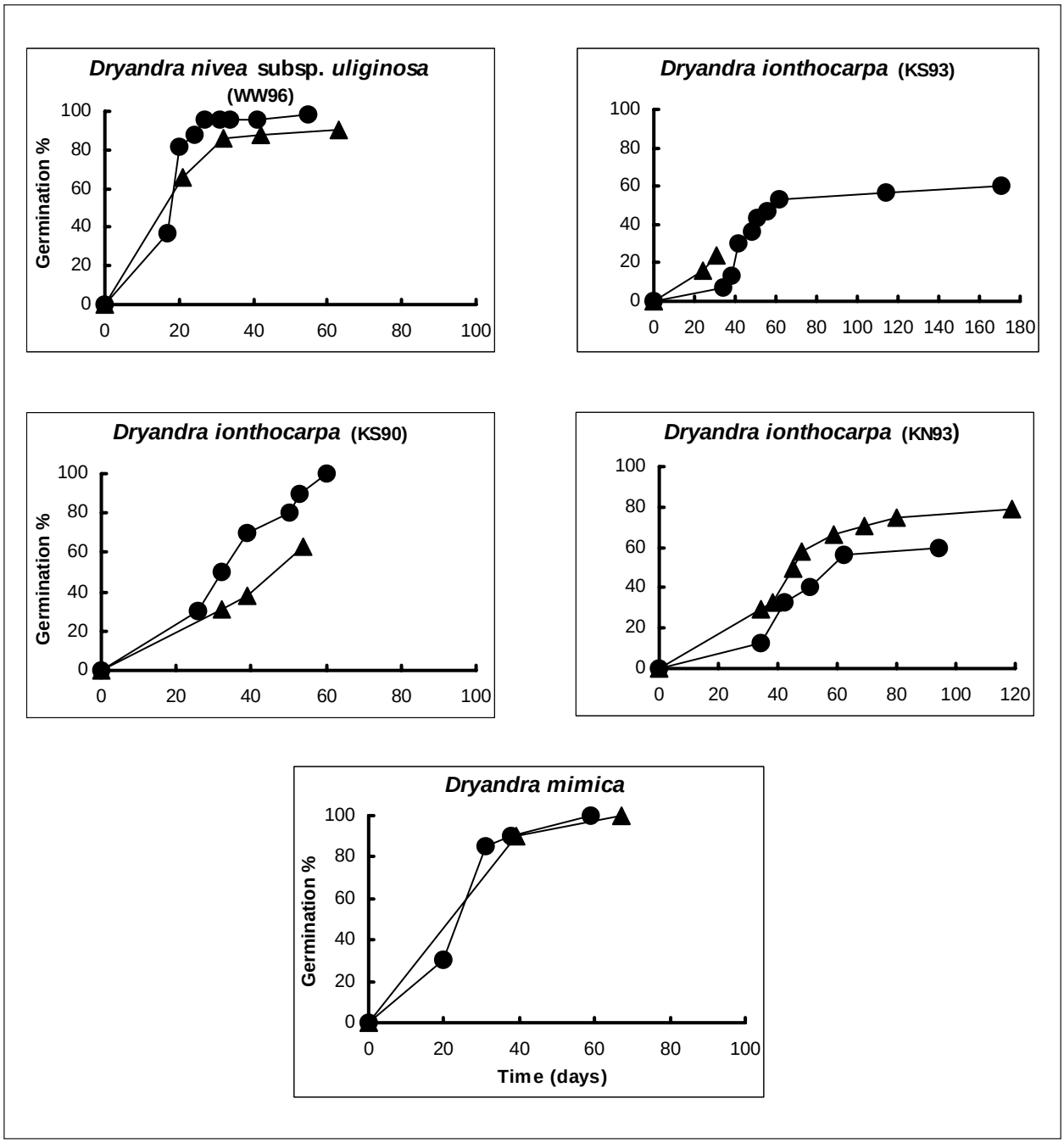

Figure 5 (continued). Cumulative germination for 13 collections demonstrating similarities in rate of germination pre- ● *and post-* ▲ *storage.*

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial assistance of Environment Australia (formerly Australian Nature Conservation Agency) for ongoing funding of this project. We would also like to thank Department of Conservation and Land Management staff, volunteers and consultants who made their time available to the Threatened Flora Seed Centre for seed collection. In particular we would like to thank Mrs. Margaret Pieroni for her help with identification of species in the field.

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