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Implications for Old-field Restoration: Diversity and abundance of arbuscular mycorrhizal fungi in soils of restored York gum (*Eucalyptus loxophleba* subsp. *loxophleba*) sites vs. remnants

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) may be critical to the restoration of ecosystem function in old-fields. Whether the diversity of the plant community is promoted by the AMF community or is the driving force of AMF diversity is unknown. We investigated two questions in the context of old-field restoration in southwestern Australia: 1) Does restoration of the plant community achieve the restoration of AMF and 2) Is AMF species diversity and abundance influenced by the plant species composition? Our study sites were located in the Ridgefield Experiment in the University of Western Australia's "Future Farm". Soil samples were collected from beneath York gum trees (Eucalyptus loxophleba subsp. loxophleba) in restored sites of varying plant species diversity and York gums in adjacent remnants. A total of 36 samples (nine per experimental treatment) were collected. AMF spores were extracted from each sample via centrifugation-sugar flotation method and examined under a dissecting microscope. Mean spore abundance of bare plot soils (331 spores/100 g soil \pm 100 spores (S.E)) differed significantly from the abundances of the other three treatment soils (ANOVA, p=0.04). AMF species richness and Shannon-Wiener diversity indices did not differ significantly among treatments. Differences in mean percent abundance of individual species were observed. We conclude that AMF species can be found in restored plant communities of old-fields and that the restoration of the AMF community in old-fields will likely depend on the restoration of the plant community in conjunction with the restoration of other abiotic factors.

Key words: restoration, mycorrhizal fungi, old-fields, species diversity

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1. INTRODUCTION

As environmental change and land degradation increase in intensity, scientists have turned to ecosystem restoration to combat these threats, aiming to reestablish important characteristics of ecosystems that promote their function. In order to achieve the restoration of ecosystem function, the interactions between species and communities within the ecosystem must be thoroughly examined. Recently, the relationship between the plant community and soil biota has come to the forefront of research on this topic. Researchers suggest that restoration projects must facilitate linkages between plants and soil biota for ecological function to be fully restored to the ecosystem (Harris et al. 2005; Kardol and Wardle 2010). Since different plant species support different communities of soil biota, it is possible that the composition of the restored site's plant community may influence soil biota recovery (Kardol and Wardle 2009). However, whether the species diversity of the plant community influences or is influenced by the diversity of soil biota is unclear and widely debated (Harris 2009).

Arbuscular mycorrhizal fungi (AMF) are key components of soil biota that may be influenced by the species diversity of the plant community and vice versa. In the context of restoration, it has been suggested that AMF and other mycorrhizal fungi could improve the growth, survival, and successful establishment of target plant species (Harris 2009). AMF usually form mutualistic symbioses with a variety of plant species that generally involve nutrient transfer between symbionts (Smith and Read 2008). AMF depend on the symbiosis to complete their life cycle and for a reliable supply of organic carbon (Smith and Read 2008). Benefits to the plant include increased drought and disease tolerance (Smith and Read 2008).

As is the case for soil biota more generally, it is unclear whether plant community diversity could also be promoted by diversity of AMF in the soil or if plant diversity is the

driving force of AMF diversity. For example, one study found that AMF sporulation of large species (e.g. *Gigaspora s.p*) increased with increasing plant diversity while that of smaller-spored species varied with host diversity, suggesting that changes in AMF diversity are responses to changes in aboveground diversity (Burrows and Pfleger 2002). Other studies have shown that greater AMF species richness is often correlated to increased plant diversity, but suggest that AMF are the determinants of plant diversity (van derHeijden et al. 1998; Vogelsang et al. 2006). Overall, researchers seem to agree that interactions exist between the two communities that maintain certain ecosystem functions (van derHeijden et al. 1998; Harris 2009; Kardol and Wardle 2010).

In particular, AMF may play a critical role in the restoration of ecosystem function to old-fields. AMF has been shown to mediate plant coexistence in early-mid successional ecosystems (reviewed by Hart et al. 2003). However, functional restoration of old-field vegetation is often limited by nutrient enrichment of the soil (Allen and Allen 1990; Johnson et al. 2003) and the loss of available AMF propagules (i.e., spores, hyphae, colonized roots) from soil disturbance during cultivation (Johnson et al. 1991). The absence of mycorrhizal plant species on early successional temperate old-fields has been associated with both nutrient enrichment and cultivation disturbance (Johnson et al. 1991).

Old-fields in the wheatbelt of Western Australia provide a unique ecosystem to examine AMF communities in restored sites and its relationship with the plant community. Similar to other ancient soils in the world, plant growth is limited by phosphorus (P) on Western Australian soil (Richardson et al. 2004). Mycorrhizal associations that increase P-uptake are one of the many mechanisms that have evolved to overcome this limitation (Boughter and Tommerup 1996; Brundrett 1991). Applications of phosphate fertilizer are required for cultivation of these soils (Beard, 1990), however, over time, increased P could negatively affect both native plants adapted to the low-P conditions and mycorrhizas (Bowen 1981; Handreck 1997; Smith and Read 1997). As the residues can remain in the soil decades after lands are no longer used for agriculture (Standish et al. 2006), the long-term adverse effects of the P may inhibit the success of restoration projects in old-fields (Standish et al. 2007).

Fundamentally, what remains unknown about the AMF communities of old-fields in Western Australia, as in other previously disturbed ecosystems, is whether the restoration of AMF to the ecosystem depends on the restoration of the plant hosts. Whether the diversity of the restored plant community affects the restoration of AMF is also unknown. In the present study, we aimed to address these two broad questions: 1) Does plant community restoration achieve the restoration of AMF and 2) is AMF diversity influenced by the plant species composition? We addressed our questions in the context of old-field restoration in the wheatbelt of Western Australia, focusing on the restoration of York gums (*Eucalyptus loxophleba* subsp. *loxophleba*), which are a dominant canopy tree in this ecosystem. We compared the AMF communities of soils beneath York gums planted in restored old-field plots with varying species diversity to those from soils beneath York gums in remnants.

We hypothesized that soils from beneath York gums in the restored sites and remnants would have similar AMF diversity and abundance because of possible host-specificity associated with AMF spores. We also hypothesized that species diversity of the plant community would have a positive correlation with AMF diversity and abundance, that is, soil from beneath York gums in a highly diverse plant community would have a high AMF diversity and abundance compared with soils from beneath York gums in a low-diversity restoration plot. Lastly, we hypothesized that soils sampled in "bare" restoration plots (i.e., no woody species planted) would contain a depauperate AMF community compared with the other samples due to the lack of woody host plants. Our research may provide insight into general mechanistic relationships between plant and AMF communities that has implications for the success of future restoration efforts that target not just York gum, but also a wide range of other plant species and ecosystems.

2. MATERIALS & METHODS

2.1 Study Sites

A) Ridgefield Multiple Ecosystem Services Experiment

The restoration study site is located within the University of Western Australia's (UWA) "Future Farm" in the wheatbelt of southwestern Australia. The Ridgefield Multiple Ecosystem Services Experiment (hereafter Ridgefield) was established in 2010 to investigate how different plant assemblages can provide additional ecosystem services to the main service of carbon sequestration (Perring et al. 2012). The experiment was initiated by the Ecosystem Restoration and Intervention Ecology (ERIE) group, which is affiliated with UWA.

Native plants, such as *Eucalyptus loxophleba* and *Acacia acuminata*, were planted in plots within Ridgefield and assembled into ten treatments (Figure 1a). The treatments reflect a gradient of species and functional group richness, and structural complexity that will change with time. A substitutive design (Fox 2005), where individuals of *E. loxophleba* were "replaced" with individuals of different species, was used for treatments involving greater functional and species richness (Figure 1c). Bare plots were included as a treatment to assess what changes will occur without deliberate plantings. The gradient of species richness in Ridgefield plots makes it a suitable site for our study because it allows us to investigate the effects of aboveground plant

diversity on belowground AMF diversity in the context of ecosystem restoration. The bare plots serve as control sites for us to examine AMF diversity in the absence of woody plants. We controlled for the variation in soil type and land-use history across the site by only sampling from plots with similar residual nutrient concentrations and the same land-use history.



Figure 1. Ridgefield Multiple Ecosystem Services Experiment (taken from Perring et al. 2012) (a) map of plot layout and species diversity; plots used in this study— C1, C2, C10, D1,D2, D10, E1, E2, and E10 (b) location of the site in Western Australia (c) representation of plantings in a plot of higher functional and species richness.

B) York gum (E.loxophleba subsp. loxophleba) Remnants

Three remnant sites within the "Future Farm" were selected on the basis of York gum prevalence. All of the sites were heavily dominated by York gum, resembling the plots with the lowest diversity of plantings in Ridgefield where York gum was the only species planted, and were surrounded by sheep-grazed farmland.

2.2 Soil Sampling

We collected soil samples from beneath York gum trees in three experimental treatments within Ridgefield—bare (control), York gum only (low-diversity site), and York gum plus seven other species (high-diversity site). Three replicate plots of each experimental treatment were selected in the formerly grazed area of the farm based on similarity in soil texture and nutrient contents (see Perring et al. 2012). We sampled soils from beneath three randomly selected trees or positions within each plot. In order to reduce the spatial heterogeneity often associated with sampling for AMF spores (Whitcomb and Stutz 2007), we sampled from three different points about 30 cm away from the trunk of each tree and bulked them for form one sample. Samples were collected with a hand trowel to a depth of about 15 cm where the density of arbuscular mycorrhizal propagules is generally greatest (Johnson et al. 1991). The same method was used to collect soil samples from beneath York gum trees in the three remnants near Ridgefield. Three trees in each remnant were randomly selected and then sampled. A total of 36 samples were collected (nine per experimental treatment) and stored in the fridge at 4°C pending spore extraction.

2.3 Spore Extraction

We described the diversity of the AMF community based on the morphology of their spores. While spore populations do not reflect the entire AMF community colonizing the plant roots because some non-sporulating AMF species may be present (Clapp et al. 1995), we believe

that using spores to characterize the AMF community of our study sites was a valid approach for our purposes.

Spores were extracted from the soil samples via the centrifugation-sugar flotation method (Walker et al. 1982). Soil samples were first thoroughly mixed and sieved to remove large rocks and other debris. We then weighed 100 g of the sieved soil for spore extraction and mixed it with DI water, letting it sit for an hour before sieving it in fractions through 250 µm, 90 µm, and 50 µm sieves. We combined the 250 µm and 90 µm fractions because our initial trials showed that very few spores were extracted from the 250 µm fraction alone. The 90 µm and 50 µm fractions were transferred to Falcon tubes and centrifuged at 2000 rpm for 5 minutes. We decanted the supernatant, resuspended the pellet in 50% sucrose solution with a specific gravity of 1.18 (480 g sugar/L), and centrifuged at 2000 rpm for 1 minute. The supernatant was decanted onto a fine sieve, washed with DI water to remove the sucrose, and the fraction was backwashed into a petri dish for microscopic examination.

2.4 Spore Classification

We examined the extracted spores from the 90 µm and 50 µm fractions of each sample separately with a dissecting microscope and counted the number of live spores, categorizing different taxa (potentially species) based on color. Pictures of spores were taken and sent to Chris Walker, a recognized expert in the AMF field, for further species identification. Classification of extracted spores is ongoing.

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2.5 Data and Statistical Analyses

Spore counts from 90 μ m and 50 μ m fractions were combined for each tree. Proportions of individual species for each plot were determined by dividing the number of spores of an individual species by the total spore count of the plot.

Total spore abundance (number of spores per 100 g of soil) for each plot was calculated as the sum of the spore counts from each tree sampled in the plot. We compared mean spore abundance of plots between all four experimental treatments with a generalized linear model and an ANOVA. We first fitted the most complicated model (i.e., spore number was a function of treatment and its interaction with block) and then simplified in order to find the minimum adequate model to explain the variation in the data. After determining that there was no significant loss in explanatory power through a model comparison with an ANOVA (p>0.05), we then assessed what factors explained the variation using a Chi-squared test (p<0.05). In dealing with count data in this analysis, we used a quasipoisson error structure to account for variance rising proportional to mean abundance and overdispersion (Crawley 2007).

Using the total spore abundance calculated for each plot, we determined the AMF diversity of each plot with the Shannon-Wiener Diversity Index. We compared the diversity indices of the experimental treatments with an ANOVA, fitting the most complicated model and then simplifying as we did with the spore abundance data. We used an F-test (p>0.05) to determine if simplification of the model was justified.

Species richness of AMF within each plot was calculated as the number of species observed across all three trees in the plot. We compared mean species richness of plots between all four experimental treatments with the same statistical tests used for mean spore abundance.

All statistical tests were conducted in R (R Core Development Team, 2012).

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3. RESULTS

3.1 AMF Spore Abundance

A total of seven spore types were found in this study, although four of them were unable to be classified into taxa. *Acaulospora sp.* (Figure 2a) was the most abundant species in soils from plots of all three experimental treatments in Ridgefield, with a mean percent abundance ranging from 44.3 to 57.8% (Table 1). *Acaulospora sp.* was found in much lower abundance in soils from York gums in the remnants with a mean percent abundance of 10.7% (Table 1) and those soils generally had a lower spore abundance than the Ridgefield soils overall, although this was not significant (Figure 3). *Funneliformis mosseae* was the second most abundant species in the samples (Figure 2c). The mean percent abundance of *Funneliformis mosseae* was greatest in the bare plot soils at 18.7% and lowest in the remnant soils at 10.9% (Table 1). *Gigaspora sp.* was the third species identified, however it was only observed in 25% of total samples examined and typically only one or two spores per sample were found (Figure 2b). The four unclassified species made up the greatest percentage of the total spore abundance in remnant soils at 77.7% and were in much lower abundance in the soils of the three treatments in Ridgefield (Table 1).

Plant species diversity of the site was found to have a statistically significant effect on mean spore abundance (ANOVA, p-0.04). Mean spore abundance was highest in soil from beneath York gums in the bare (control) plots at 331 spores/100 g soil \pm 100 spores (S.E). Soils from beneath York gums in the three other treatments—"York gum + Other", "York gum only", and remnant—did not differ significantly in mean spore abundance from each other, but did from the abundance in the bare plot soils (Figure 3).



Figure 2. AMF spores extracted from soil samples. (a) *Acaulospora sp.* (b) *Gigaspora sp.* (c) *Funneliformis mosseae-* shown by arrow (d) Unclassified clear spore



Figure 3. Mean spore abundance of soils from beneath York gums (YG) in Ridgefield plots and remnants. Error bars represent \pm S.E. Letters above bars indicate statistically different means based on a Chi-squared test from a generalized linear model with quasipoisson errors. Plant species diversity of plots was found to have a statistically significant effect on spore abundance (ANOVA, p=0.04). Mean spore abundance of soils from bare (control) plots was nearly twice as great as that of soils from "YG only plots" and three times as great as that of soils from the "YG + Other" plots and the remnants.

Treatment	Species Richness	Shannon- Weiner Diversity Index ^b	% Acaulospora sp. ^b	% Funneliformis mosseae ^b	% Gigaspora sp. ^b	% Unclassified ^a
Bare	6	1.39 ± 0.10	57.8 ± 3.1	18.7 ± 2.7	0.4 ± 0.3	23.0 ± 3.5
YG + Other	6	1.33 ± 0.06	49.1 ± 3.9	11.6 ± 2.0	0.5 ± 0.5	38.8 ± 2.1
YG only	6	1.34 ± 0.03	44.3 ± 12.5	16.5 ± 4.8	0.4 ± 0.4	38.8 ± 17.2
Remnant	5	1.46 ± 0.05	10.7 ± 5.34	10.9 ± 5.2	0.7 ± 0.4	77.7 ± 10.2

Table 1. Summary of mean species richness, mean Shannon-Weiner diversity indices, and mean % abundance of individual species in soils from each treatment

^a Includes four species

 $b \pm S.E$

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3.2 AMF Species Richness

The bare, "YG + Other", and "YG only" soils had a mean AMF species richness of six, whereas that of the remnant soils was five (Table 1). There was no significant difference in mean species richness among the experimental treatments (ANOVA, p>0.05), even if the four unclassified species were not considered in the analysis. The remnant soils had the highest mean Shannon-Wiener diversity index at 1.46 (Table 1), however we did not find a significant difference in mean diversity indices among experimental treatments (ANOVA, p>0.05).

4. DISCUSSION

Our study has shown that AMF species can be found in restored plant communities. The presence or absence of vegetation appears to influence AMF spore abundance, however it is unclear from our results whether the species diversity of the vegetation is a significant determinant as well. The variation in AMF species diversity among experimental treatments was not found to be significant despite some differences in percent abundance of individual species therefore we cannot conclude that plant species diversity influences AMF species diversity.

4.1 Greater AMF spore abundance in soils taken from bare plot soils

Contrary to our hypotheses, the greatest total abundance of AMF spores was observed in bare plot soils, while the total abundance of AMF spores did not differ between soils from the other experimental treatments. A previous study reported that there were 30 to 150% more spores in 16-species plots than in one-species plots (Burrows and Pfleger 2002), suggesting that we would have expected to see a much greater abundance of AMF spores in soils from beneath York gums in the "York gum + Other" treatment than those from York gums in the "York gum only" treatment.

The predominance of weeds in the bare plots may account for the high total abundance of AMF spores in those soils. While the experimental plots in Ridgefield primarily consisted of planted tree species, the bare plots were covered in various weed species. A more diverse assemblage of weeds could have fostered greater sporulation of the AMF species that we were able to identify. The lack of greater diversity in the remnants, which were dominated by York gum and appeared to have relatively homogenous ground cover that consisted mainly of leaf litter, may explain the difference in total abundance of AMF spores between remnant soils and bare plot soils.

That we did not observe a pattern of increasing spore abundance with increasing plant diversity may be attributed to several factors. Firstly, soils from both treatments, "York gum + Other" and "York gum only", have a similar land-use history. The plots are located in the formerly grazed area of the farm and likely experienced similar levels of agricultural disturbance. While such disturbance has been shown to reduce AMF diversity and affect the composition of the spore assemblage, it does not affect spore abundance as negatively. Spores of some species are capable of long-term survival in soil and can germinate repeatedly in the absence of host-plant roots (Smith and Read 2008). Secondly, the age of the plantings in both treatments is the same. Assuming that due to similar land-use history the soils had a similar baseline of AMF spore abundance and/or diversity, then colonization of the York gum seedlings would likely have occurred at the same rate. We only sampled soils from beneath York gums so it is possible that plant species diversity does affect AMF spore abundance, but such effects could not be detected

in this study because the plantings are still relatively young and perhaps mature hyphal networks have not yet established.

4.2 Similar AMF species richness between restored plots and remnants

While we found a difference in AMF spore abundance among experimental treatments, AMF species richness did not differ significantly. Our results corroborate those of a previous study that compared the AMF diversity of soils with different land-use histories, finding that AMF species richness was similar in old-field soil to never-cultivated soil (Li et al. 2007). The authors interpreted that the similarity in AMF community composition of old-field and nevercultivated soil suggested that agricultural disturbance affects the community, but a combination of natural and artificial restoration of the plant community could produce a shift towards resemblance of never-cultivated soil in old-fields over time (Li et al. 2007). Another study showed that AMF diversity did not differ between old-fields and remnants of the previous ecosystem at a local site scale (Picone 2000).

4.3 Similar diversity indices between restored sites and remnants, but different proportions of individual species

We did not find a significant difference in the diversity indices between experimental treatments. However, there are some intriguing differences in the proportional abundance of individual species between plots and treatments. The bare, "York gum + Other", and "York gum only" plots have relatively similar abundances of individual species. It is clear that the dominant species found in the plots was *Acaulospora sp.* and there are similar proportions of the other six species found. The remnants, on the other hand, have very different proportions of individual

species from the other treatments. A drastic reduction in *Acaulospora sp.* and a predominance of some of the species that were in very low abundance in the plots of other treatments is the most obvious difference. The reduction in *Acaulospora sp.* in remnants contrasts with the reports of such reductions in areas experiencing high agricultural disturbance (Smith and Read 2008), a characteristic generally not attributable to remnants. The maturity of the York gums in the remnants may offer the best explanation of our results. Other *Eucalyptus* species have been shown to exhibit a greater dependence on ectomycorrhizal fungi rather than arbuscular with maturity (Lapeyrie et al. 1985; Chilvers et al. 1987), suggesting that older York gums may form associations with ectomycorrhizal fungi rather than AMF species that tend to associate with younger trees.

Other factors affecting the results

The adequacy of our sampling is an important factor in the interpretation of our results. Based on species accumulation curves for each treatment (Appendix 1A), it is questionable whether or not we have sampled adequately for all treatments. We may have underestimated AMF diversity in these soils, given that we have only assessed diversity based on AMF spores and not taken into account non-sporulating AMF species or other AMF structures. As previous studies have shown that AMF diversity and abundance may vary with seasonality (Douds and Millner 1999), it is also possible that our study could not capture the entire AMF diversity within the plots due to the time of sampling.

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CONCLUSIONS

Given the numerous abiotic factors affecting the AMF community in the soil, it is likely that restoration of the AMF community in old-fields will not be solely dependent on the restoration of the plant community. Plant species diversity may be influential in determining AMF diversity and abundance in conjunction with other ecosystem characteristics, such as nutrient availability, seasonality, and level of disturbance over time. More extensive and longterm studies that take such characteristics into account would provide greater insight into the effects of plant species diversity on the AMF community than this study has been able to demonstrate. However, the presence of AMF in the restored plots that we sampled bodes well for the establishment of essential linkages between the plant and AMF communities in the future.

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APPENDIX

A) Species accumulation curves





Processed (Date)	Block	Diversity	Plot	Tree	Pale Yellow (Species 1)	Clear Yellow (Species 2)	Funneliformis mosseae	Acaulospora sp.	Clear (Species 3)	Sm. Cloudy White (Species 4)	Gigaspora sp.	Total # of Spores	Species Richness
X (4/5)	С	Bare	43	3	5		45	94				144	3
^ (4/23)	С	Bare	43	6	12	6	10	21	9	5		63	6
^ (4/16)	С	Bare	43	7		7	1	12	13			33	4
x (4/16)	D	Bare	46	2	5	6	25	84	10	6		136	6
x (4/10)	D	Bare	46	3	69		3	153	18		1	244	5
x (4/18)	D	Bare	46	7	12	14	46	66	7	6		151	6
x (4/11)	Е	Bare	61	2	14	1	24	97	6			142	5
x (4/23)	Е	Bare	61	4	1	1	6	19	3	4	2	36	7
x (4/16)	Е	Bare	61	5	1	1	12	25	5			44	5
x (4/18)	С	YG all	22	2	3	6	5	25	7	5		51	6
^ (4/10) V	С	YG all	22	3	1	7	8	95	18			129	5
^ (4/18)	С	YG all	22	5		5	5	7	24	3		44	5
X (4/5) X	D	YG all	25	1		2	2	31	18			53	4
(4/23) X	D	YG all	25	5	1	2	1	9	4	1		18	6
(4/16) X	D	YG all	25	8		1	12	7	10			30	4
(4/18)	Е	YG all	63	1			2	9	3			14	3
^ (4/23) ×	Е	YG all	63	2			2	10	2	2		16	4
^ (4/12)	Е	YG all	63	4	9		4	11	11	2	1	38	6
(4/10)	С	YG only	34	1	1	9	14	45	16		1	86	6
(4/16) X	С	YG only	34	2	1	2	19	27	2		1	52	6
(4/23)	С	YG only	34	8	1	8	10	66	2	3	1	91	7

Х													
(4/11) X	D	YG only	39	1	1	1	5	4	50			61	5
(4/23)	D	YG only	39	2			0	7	2	2		11	4
^ (4/16)	D	YG only	39	3	3	8	2	8	4			25	5
× (4/11) ×	Е	YG only	59	3	3	1	22	47	6			79	5
^ (4/23) V	Е	YG only	59	5	16	5	13	75	4	11		124	6
^ (4/16) ×	Е	YG only	59	7	4		21	5	6			36	4
^ (4/18) ×	Remnant	Remnant	3	2	3		1	2	2	5		13	5
^ (4/12) V	Remnant	Remnant	3	4	42		9	6	29		1	87	5
^ (4/23) ×	Remnant	Remnant	3	6	1	4	1	3	4	8		21	6
^ (4/23) ×	Remnant	Remnant	4A	2			1	5				6	2
^ (4/12) ×	Remnant	Remnant	4A	4	1		3		3	13		20	4
^ (4/18) V	Remnant	Remnant	4A	7			2	1				3	2
^ (4/23) V	Remnant	Remnant	4B	1	2	6	0	1	2	8		19	6
^ (4/18) ×	Remnant	Remnant	4B	5		7	5	3	15	5	2	37	6
^ (4/12)	Remnant	Remnant	4B	6	1	26	0		60	23		110	5