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Resistance to root-lesion nematode *Pratylenchus neglectus* identified in a new collection of two wild chickpea species (*Cicer reticulatum* and *C. echinospermum*) from Turkey

Hannah E. Rostad[1] | Roslyn A. Reen[1] | Michael H. Mumford[2] | Rebecca S. Zwart[1] | John P. Thompson[1]

[1]Centre for Crop Health, University of Southern Queensland, Toowoomba, Queensland, Australia

[2]Department of Agriculture and Fisheries, Leslie Research Facility, Toowoomba, Queensland, Australia

Correspondence
Hannah E. Rostad, Centre for Crop Health, University of Southern Queensland, Toowoomba, QLD, Australia.
Email: Hannah.Rostad@usq.edu.au

Abstract

Chickpea (*Cicer arietinum*) is a major legume crop, with Australia being the second largest producer worldwide. *Pratylenchus neglectus* is a root-lesion nematode that invades, feeds and reproduces in roots of pulse and cereal crops. In Australia, chickpea and wheat (*Triticum aestivum*) are commonly grown in rotation and annual damage by *P. neglectus* accounts for large economic losses to both crops. Cultivated chickpea has narrow genetic diversity that limits the potential for improvement in resistance breeding. New collections of wild chickpea species, *C. reticulatum* and *C. echinospermum*, have substantially increased the previously limited world collection of wild *Cicer* germplasm and offer potential to widen the genetic diversity of cultivated chickpea through the identification of accessions with good resistance. This research assessed 243 *C. reticulatum* and 86 *C. echinospermum* accessions for response to *P. neglectus* in replicated experiments under controlled glasshouse conditions from 2013 and 2014 collection missions that were received, tested and analysed in two experimental sets. Multi-experiment analyses showed lower *P. neglectus* population densities in both sets of wild *Cicer* accessions tested than Australia's elite breeding cultivar PBA HatTrick at the significance level $p < 0.05$. Provisional resistance ratings were given to all genotypes tested in both experimental sets, with *C. reticulatum* accessions CudiB_008B and Kayat_066 rated as resistant in both Set 1 and Set 2. New sources of resistance to *P. neglectus* observed in this study can be introgressed into commercial chickpea cultivars to improve their resistance to this nematode.

KEYWORDS
Cicer arietinum, *Cicer echinospermum*, *Cicer reticulatum*, crop wild relatives, *Pratylenchus neglectus*, root-lesion nematode

1 | INTRODUCTION

Chickpea (*Cicer arietinum*) is important in cereal–pulse cropping systems in Australia and worldwide, with global production of pulses increasing by over 20 million tonnes in the last decade (Belhassen et al., 2019). Chickpea plays an important role in feeding the populations of many countries while supplying a myriad of health benefits to consumers, such as high protein content, important nutrients for a balanced diet and reduction of cholesterol and the risk of cardiovascular disease (Jukanti et al., 2012).

Pratylenchus neglectus is a migratory, endoparasitic, vermiform root-lesion nematode (RLN) that invades, feeds and reproduces in the roots of many cereal and pulse crops, including chickpea. Symptoms of infestation include root necrosis and reduced root branching, which results in the reduction of crop yield (Oldach et al., 2014). *P. neglectus* reproduces via mitotic parthenogenesis with five life stages—egg, juvenile stages J2, J3 and J4, and adult—with the full life cycle completed in 4–6 weeks depending on food source availability and optimum environmental conditions (Thompson et al., 2017). *P. neglectus* is considered one of the most widespread *Pratylenchus* spp. worldwide (Behmand et al., 2019) due to its broad host range on the world's most economically important crops (Oldach et al., 2014). It is a major pathogen in the Australian grain regions where chickpea is commonly grown in rotation with wheat (Doughton et al., 1993; Reen et al., 2019; Thompson et al., 2011). *P. neglectus* causes a loss in production valued at A$73 million/year in wheat (Murray & Brennan, 2009) and A$9.6 million/year in chickpea (Murray & Brennan, 2012). Nearly all current Australian commercial chickpea cultivars are rated as moderately resistant to moderately susceptible (MR-MS) to *P. neglectus* (Matthews et al., 2021), where resistance is defined as the effects of host genes to prevent nematode multiplication in a host species (Trudgill, 1991). Resistant plants will also reduce nematode reproduction to significantly less than that of its initial population. Limited variation in the resistance ratings of existing commercial chickpea cultivars is strong evidence that sources of good resistance and genetic diversity are needed.

Cultivated chickpea has low genetic diversity because of four genetic bottlenecks: (a) a limited centre of geographic origin, namely, an area spanning modern-day north Syria and south-east Turkey; (b) the founder effect associated with domestication from a limited number of plants; (c) a change early in the crop's history from winter to spring sowing; and (d) a switch from diverse landraces to a narrower range of elite cultivars developed through modern plant breeding (Abbo et al., 2003). This makes genetic improvement of chickpea challenging. A study by von Wettberg et al. (2018) estimated that 93.5%–97.5% of the genetic variation that is found in wild *Cicer* spp. is absent in modern cultivated chickpea.

Among the annual wild *Cicer* spp., the progenitor of cultivated chickpea, *C. reticulatum*, is wholly compatible with *C. arietinum* for breeding (Coyne et al., 2020). *C. echinospermum* is the only other annual wild *Cicer* species that is cross compatible with cultivated chickpea without the use of advanced hybridization techniques (Croser et al., 2003). Therefore, these two wild *Cicer* species can be used in breeding programmes to increase the genetic diversity of cultivated chickpea and maintain crop productivity (Rao et al., 2007). Resistance has been found in *C. reticulatum* and *C. echinospermum* to major biotic and abiotic constraints to chickpea, such as the RLN *Pratylenchus thornei* (Reen et al., 2019; Thompson et al., 2011), Ascochyta blight (*Ascochyta rabei*; Devadas et al., 2005; Newman et al., 2021), grey mould (*Botrytis cinerea*; Devadas et al., 2005), Phytophthora root rot (*Phytophthora medicaginis*; Knights et al., 2008), pod borer (*Helicoverpa armigera*; von Wettberg et al., 2018), leaf miner (*Liriomyza brassicae*) and tolerance of cold and drought (Talip et al., 2018). Multiple biotic resistance is desirable for crop breeding and provides protection from one disease dominating at sites where there are multiple biotic pressures (Wiesner-Hanks & Nelson, 2016).

A 2011 study by Thompson et al. showed that *C. reticulatum* and *C. echinospermum* were largely more resistant to *P. neglectus* or *P. thornei* than commercial chickpea cultivars tested or had dual resistance to both species. However, only a small number of wild *Cicer* accessions was screened in that study because of the small numbers of original accessions of wild *Cicer* located in global genebanks at that time: 18 unique accessions of *C. reticulatum* and 10 of *C. echinospermum* (Berger et al., 2003). To address the limited number of wild *Cicer* accessions available in global genebanks, scientists from Australia, the United States and Turkey conducted a series of comprehensive collection expeditions in south-east Turkey spanning the years 2013–2018 (Toker et al., 2021). These recent collection missions attempted to cover the full geographic distribution of *C. reticulatum* and *C. echinospermum* in south-east Turkey. The 2013 collection mission focused on targeting areas with known latitudinal and longitudinal data from the original collection as a starting point for locating *Cicer* spp. in south-east Turkey. The 2014 collection mission largely focused on information gathered from local shepherds and finding similar growth habitats of already located wild *Cicer* in further eastern regions of Turkey (Toker et al., 2021; von Wettberg et al., 2018). These new collections are an integral resource for international research to identify new sources of genetic resistance in wild chickpea germplasm to biotic and abiotic constraints and facilitate cultivated chickpea improvement worldwide (Reen et al., 2019; Talip et al., 2018; von Wettberg et al., 2018).

Twenty-six accessions from this new collection, 20 *C. reticulatum* and six *C. echinospermum*, were selected by von Wettberg et al. (2018) to represent the genetic and environmental diversity of the 2013 wild *Cicer* collection. Nested association mapping (NAM) populations have been developed by crossing these 26 wild *Cicer* accessions with a common elite parent from each of the major chickpea growing regions of the world, namely, Turkey, Canada, the USA, Ethiopia, India, Israel and Australia (von Wettberg et al., 2018). The elite cultivar chosen to be representative of Australian chickpea cultivars was the moderately resistant cv. PBA HatTrick. At this time there are no Australian chickpea cultivars rated as fully resistant.

This study aimed to screen a new and wider collection of wild *Cicer* accessions for *P. neglectus* resistance using final nematode population densities and known resistance responses to commercial

chickpea cultivars and current Australian breeding lines. New sources of *P. neglectus* resistance will widen the genetic base of cultivated chickpea, which in turn will reduce nematode populations in infested sites, improve yields and provide a resilient crop to strengthen rotation choices in Australia and globally.

2 | MATERIALS AND METHODS

2.1 | Plant material

A total of 329 wild *Cicer* accessions (243 *C. reticulatum* and 86 *C. echinospermum*) were obtained from the Australian Grains Genebank (AGG; Horsham, Victoria). Accessions tested in this study were collected from 32 sites within seven provinces of south-east Turkey (Figure 1; Table 1). Thirty-nine reference genotypes with known responses to *P. neglectus* were included in the experiments, comprising 19 Australian chickpea cultivars (Table 2), one Indian chickpea breeding line, 10 Australian chickpea breeding lines derived from wild *Cicer*, six wild *Cicer* accessions from the original genebank collections (Table 3), and five reference hexaploid wheat lines (Table 2). The reference wheat cultivars, with known resistance and susceptibility ratings, were included in the experiments as confirmation that *P. neglectus* multiplied as expected.

2.2 | Experimental design

Glasshouse experiments were tested in two sets as wild *Cicer* accessions were released from quarantine after import into Australia. Each accession was tested at least twice across four experiments. In 2016, 174 accessions (133 *C. reticulatum* and 41 *C. echinospermum*)

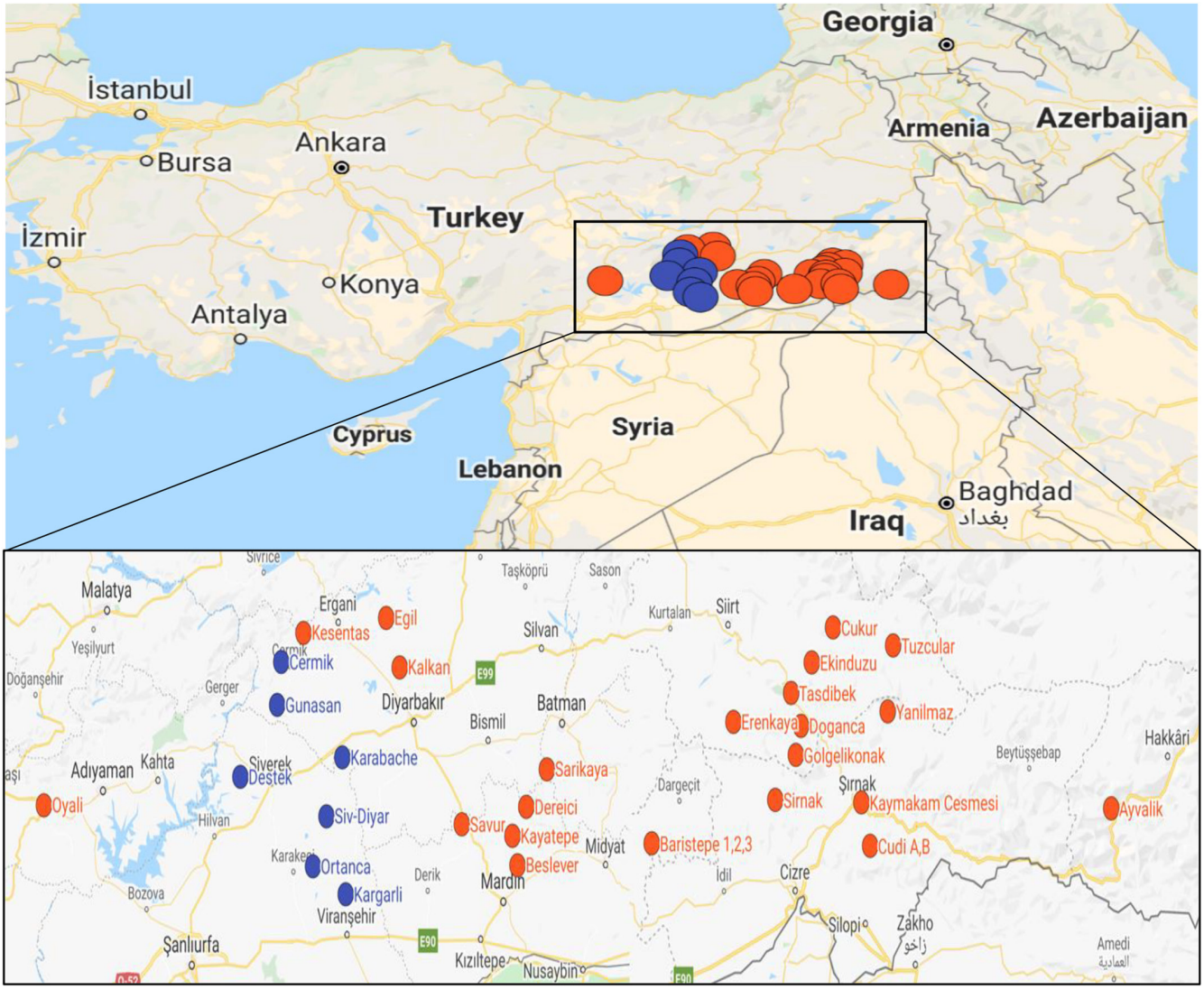

FIGURE 1 Map of south-east Turkey and the 32 collection sites where *Cicer reticulatum* and *C. echinospermum* accessions were collected (accessions from 21 collection sites tested in Experiments 1 and 2 plus an additional 11 collection sites tested in Experiments 3 and 4). Map data obtained from Map Maker 2021. Red circles, *C. reticulatum*; blue circles, *C. echinospermum*

TABLE 1 Passport information on the wild *Cicer* accessions tested for *Pratylenchus neglectus* resistance showing province and collection site, species, number of accessions at each collection site (*n*), prefix used with accession code number and year of collection

Province	Collection site	Species	*n*	Prefix	Accession code number	Year of collection
Adiyaman	Oyali	*C. reticulatum*	11	Oyali	071, 073, 076, 081, 084, 085, 100, 101, 104, 105, 107	2013
Diyarbakir	Cermik	*C. echinospermum*	6	Cermi	061, 063, 071, 072, 073, 075	2013
	Egil	*C. reticulatum*	7	Egil	063, 065, 066, 072, 073, 074, 075	2013
	Gunasan	*C. echinospermum*	4	Gunas	061, 062, 100, 101	2013
	Kalkan	*C. reticulatum*	7	Kalka	061, 064, 065, 066, 067, 070, 074	2013
	Kesentas	*C. reticulatum*	12	Kesen	062, 065, 066, 067, 071, 072, 073, 074, 075, 077, 101, 104	2013
Hakkari	Ayvalik	*C. reticulatum*	1	Olgun	026	2014
Mardin	Baristepe 1	*C. reticulatum*	8	Bari1	062, 063, 064, 068, 069, 091, 092, 093	2013
	Baristepe 2	*C. reticulatum*	5	Bari2	062, 064, 067, 072, 074	2013
	Baristepe 3	*C. reticulatum*	18	Bari3	064, 065, 067, 072C, 073, 074, 075, 079, 091, 092, 100, 101, 102, 103, 104, 106D, 110, 112	2013
	Beslever	*C. reticulatum*	8	Besev	061, 062, 065, 066, 074, 075, 079, 083	2013
	Dereici	*C. reticulatum*	10	Derei	062, 065, 066, 069, 070, 072, 073, 074, 075, 078	2013
	Kayatepe	*C. reticulatum*	10	Kayat	061, 063, 064, 066, 067, 070, 071, 077, 080, 081	2013
	Sarikaya	*C. reticulatum*	13	Sarik	061, 063, 064, 065, 066, 067, 072, 073, 074, 077, 078, 080, 081	2013
	Savur	*C. reticulatum*	1	Savur	063	2013
Siirt	Cukur	*C. reticulatum*	1	Golko	001	2014
	Doganca	*C. reticulatum*	18	Dogan	026, 027, 028, 029, 031, 032, 033, 034, 035, 036, 037, 038, 039, 040, 042, 043, 044, 045	2014
	Ekinduzu	*C. reticulatum*	17	Ekind	043, 044, 045, 047, 048, 049, 050, 051, 052, 053, 054, 055, 056, 057, 058, 059, 060	2014
	Erenkaya	*C. reticulatum*	2	Erenk	001, 002	2014
	Golgelikonak	*C. reticulatum*	9	Golge	026, 031, 032, 034, 035, 036, 037, 038, 039	2014
	Tasdibek	*C. reticulatum*	1	Tasdi	025	2014
	Tuzcular	*C. reticulatum*	5	Tuzca	032, 035, 038, 039, 044	2014
	Yanilmaz	*C. reticulatum*	1	Yanil	013	2014
Sirnak	Cudi A	*C. reticulatum*	18	CudiA	101A, 102, 103C, 104, 105, 107, 109, 122, 124, 125, 127, 128, 151, 152, 153, 154, 155, 221	2013
	Cudi B	*C. reticulatum*	14	CudiB	004, 005, 006, 008B, 009, 011, 012, 015, 016, 017, 018, 019, 022C, 023	2013
	Kaymakam Ceşmesi	*C. reticulatum*	4	Kayma	005, 035, 039, 044	2014
	Sirnak	*C. reticulatum*	42	Sirna	060, 061, 063, 064, 066, 067, 069, 070, 071C, 081B, 082, 083, 084, 085, 087, 088, 089B, 090, 101, 103, 104, 105, 110, 111A	2013
					030, 032, 034, 035, 036, 037, 038, 039, 040, 041, 042, 043, 044, 046, 047, 050, 051, 052	2014
Urfa	Destek	*C. echinospermum*	12	Deste	061, 063, 064, 066, 071, 072, 073, 075, 077, 078, 079, 080	2013
	Karabahce	*C. echinospermum*	17	Karab	062, 063, 066A, 067, 081, 082, 084, 085C, 086, 091B, 092, 093, 162, 164, 171, 172, 174	2013
	Kargali	*C. echinospermum*	33	Isoha	002, 010, 013, 018, 024, 025, 026, 027, 028, 030, 031, 032, 033, 034, 036, 037, 038, 039, 040, 042, 043, 044, 045, 046, 047, 048, 049, 050, 051, 052, 053, 054, 055	2014
	Ortanca	*C. echinospermum*	2	Ortan	061, 066	2013
	Siv-Diyar	*C. echinospermum*	12	S2Drd	061, 062, 065, 100, 101, 102, 104, 105, 106, 107B, 108, 109	2013

TABLE 2 Commercial chickpea and wheat used as reference cultivars

Species	Cultivar	Resistance rating[a]
Cicer arietinum	PBA Boundary	RMR
	Flipper	RMR
	Howzat	RMR
	PBA Pistol	RMR
	Sona	RMR
	Sonali	RMR
	Jimbour	MR
	PBA Drummond	MR
	PBA HatTrick	MR
	Yorker	MR
	Almaz	MR-MS
	Genesis 090	MR-MS
	Kyabra	MR-MS
	Moti	MR-MS
	Neelam	MR-MS
	PBA Maiden	MR-MS
	PBA Seamer	MR-MS
	PBA Slasher	MR-MS
	PBA Striker	MR-MS
Triticum aestivum	Yenda	MR
	Wyalkatchem	MR-MS
	Machete	S
	Brookton	S
	Gregory	S

[a]Resistance ratings: RMR, resistant-moderately resistant; MR, moderately resistant; MR-MS, moderately resistant-moderately susceptible; S, susceptible (Matthews et al., 2021).

from the 2013 collection were received and tested as Set 1 (Experiments 1 and 2, conducted in 2016 and 2017, respectively) at the Leslie Research Facility, Toowoomba, QLD (27.56°S, 151.95°E). The accessions were distributed across 21 collection sites in five Turkish provinces: Adiyaman, Diyarbakir, Mardin, Sirnak and Urfa. In 2018, an additional 155 accessions (110 *C. reticulatum* and 45 *C. echinospermum*) from the 2013 and 2014 collections were received and tested as Set 2 (Experiments 3 and 4, conducted in 2019 and 2020, respectively) at the University of Southern Queensland, Toowoomba, QLD. These accessions were distributed across 32 collection sites in seven Turkish provinces: Adiyaman, Diyarbakir, Hakkari, Mardin, Siirt, Sirnak and Urfa. A subset of 11 *C. echinospermum* and 36 *C. reticulatum* accessions from Set 1, ranging in response to *P. neglectus*, were also included in Experiments 3 and 4. An unplanted control treatment was included in each experiment to establish a baseline for *P. neglectus* population densities. All treatments were tested for response to *P. neglectus* with three replicates for each treatment, randomized using a row-column experimental design for each experiment.

2.3 | Glasshouse conditions

Experiments were carried out in the Australian winter to spring period of July–November under controlled glasshouse conditions. Screening for resistance is ideally conducted under glasshouse conditions where environmental influences that affect nematode reproduction such as temperature, soil moisture and inoculation rate can be controlled (Nombela & Romero, 1999). Air temperature was maintained at around 20–25°C and soil temperature was maintained at around 22°C, which is optimum for *P. neglectus* reproduction (Vanstone & Nicol, 1993), using under-bench heating (Thompson et al., 2017). Plants were grown on a capillary matting bottom-watering system set at 6 cm water tension by a float-valve system to ensure optimum water supply during the growing period. This continuous supply of water to the soil allows for favourable nematode movement, feeding and reproduction (Castillo & Volvas, 2007), which is important to obtain accurate phenotypic data in resistance testing. Plants were grown in square pots measuring 70 mm wide and 150 mm high, suitable for bottom watering (Garden City Pots). Pots contained 330 g (oven-dry [OD] equivalent) of black vertosol soil (Isbell, 1996) of the Waco series (Beckmann & Thompson, 1960), which had been pasteurized at 85°C for 45 min. Fertilizer in solution was added to 80% of the total soil volume providing 200 mg/kg nitrate nitrogen, 25 mg/kg phosphorus, 88 mg/kg potassium, 36 mg/kg sulphur and 5 mg/kg zinc.

Wild *Cicer* seed was scarified with a scalpel, removing a section of testa from the non-germ end to facilitate germination, and placed in moistened soil in the pots. A 1 ml slurry of Rhizobium Group N *Mesorhizobium ciceri* strain CC1192 (in a peat carrier) (Queensland Agricultural Seeds) was inoculated into each pot at planting. A pure culture of *P. neglectus* was multiplied on susceptible wheat for 16 weeks in the glasshouse before extraction. Nematodes were extracted using the Whitehead tray method (Whitehead & Hemming, 1965), by spreading the soil and roots evenly on a slightly raised grated basket lined with Kimwipes (Kimtech), sitting in a tray with 1 L water. For inoculum production, samples were left for up to 120 h at 22°C. After this period, nematodes were collected by pouring the water and nematodes at an angle through a 20-μm aperture sieve to obtain a suspension of nematodes in water, which was stored at 4°C until microscopic enumeration. A 10 ml aliquot of the nematode suspension was pipetted around the seed at planting, supplying 3300 *P. neglectus* per pot, equivalent to 10 *P. neglectus*/g soil. The remaining 20% soil volume was added to the pot as a cap after sowing and inoculation.

2.4 | Plant harvest, nematode extraction and enumeration

After 16 weeks, water supply to the plants was stopped and after a drying period of 2–3 days the plants were harvested. This drying period resulted in optimum soil moisture content (c.45%) for processing of the soil and roots for nematode extraction. Soil from individual pots was manually processed, roots cut into approximately 10 mm pieces, and the whole sample mixed to homogenize. Subsamples of 150 g for

TABLE 3 Wild *Cicer*-derived breeding lines and *Cicer reticulatum* and *C. echinospermum* accessions from the original genebank collection

Species	Line/accession	Wild *Cicer* derivation	Country
Cicer arietinum	CICA0709	L204 *C. echinospermum*	Australia
	CICA1314	ILWC246 *C. echinospermum*	Australia
	CICA1317	ILWC104 *C. reticulatum*	Australia
	CICA1421	ILWC104 *C. reticulatum*	Australia
	CICA1427	ILWC246 *C. echinospermum*	Australia
	00283–1095–1002	ILWC140 *C. reticulatum*	Australia
	D05222>F3TMWR2AB001	ILWC140 *C. reticulatum*	Australia
	D05253>F3TMWR2AB001	ILWC140 *C. reticulatum*	Australia
	D05293>F3TMWR2AB002	ILWC246 *C. echinospermum*	Australia
	D05295>F3TMWR2AB026	ILWC246 *C. echinospermum*	Australia
	ICC11323	Landrace	India
Cicer reticulatum	ILWC 140		Turkey
	ILWC 127		Turkey
	ILWC 115		Turkey
Cicer echinospermum	PI 527932		Turkey
	ILWC 39		Turkey
	ILWC 180		Turkey

nematode extraction and 100 g soil for gravimetric moisture content were taken. Soil gravimetric moisture content was determined by drying in a forced draught oven for 48 h at 105°C. Nematodes were extracted using the method described above, adjusted slightly for the smaller volume of soil being extracted. The 150 g subsample of soil and roots was spread evenly on a slightly raised grated basket lined with facial tissues (Kleenex), sitting in a tray with 1 L water for 48 h at 22°C. After the 48-h period, nematodes were collected by pouring the water and nematodes at an angle through a 20-μm aperture sieve obtaining approximately 15 ml water suspension of nematodes, which was stored in 30-ml vials at 4°C until microscopic enumeration. Reen and Thompson (2009) demonstrated that the efficiency of this Whitehead tray method for 48 h and sieving with a 20-μm aperture sieve recovers 70% of *Pratylenchus* populations, which is sufficient to show differences in population densities for resistance testing. *P. neglectus* were counted in a 1-ml Peters slide (Peters, 1952; Chalex corporation) under a compound microscope (40×; BX53, Olympus). Nematode population densities were expressed as number of *P. neglectus*/kg soil and roots and will be reported from here on as *P. neglectus*/kg.

2.5 | Statistical analysis

The analysis of *P. neglectus* population densities (per kg) was performed using two multi-experiment analyses, the first consisting of Experiments 1 and 2, and the second consisting of Experiments 3 and 4. The decision to separate the analysis of the experiments in this way was made based on both practical and statistical considerations, discussed below.

The analysis of both sets of experiments was conducted using a linear mixed model framework, whereby *P. neglectus* population densities required a log transformation to meet the model assumption of homogeneity of variance, a commonplace transformation implemented in the analysis of RLN due to their exponential population growth (Proctor & Marks, 1974). The general form of the linear mixed model for both sets of analyses is as follows:

$$\log\left(y_{ijkl}\right) = c + s_k + d_j + sd_{jk} + u_{ijk} + b_{jl} + e_{ijkl}, \quad (1)$$

where y_{ijkl} is the *P. neglectus* population density (per kg) for genotype i nested within species k, in experiment j, in replicate l; c is the overall constant; s_k is the fixed effect for the k^{th} species, where species consisted of five levels (*T. aestivum*, *C. arietinum*, *C. echinospermum*, *C. reticulatum* and unplanted); d_j is the fixed effect for the j^{th} experiment; sd_{jk} is the interaction effect between the j^{th} experiment and k^{th} species, and u_{ijk} is the genotype-by-experiment (G × E) interaction effect for the i^{th} genotype nested within the k^{th} species for the j^{th} experiment. The replicate block effect for the l^{th} replicate block within the j^{th} experiment is represented by b_{jl}, and e_{ijkl} is the error term for each individual pot.

Random effects were fitted for the genotype ×experiment interaction, the replicate block effects, and the error terms such that

$$E\left(u_{ijk}\right) = E\left(b_{jl}\right) = E\left(e_{ijkl}\right) = 0 \quad (2)$$

$$var(u_{ijk}) = \sigma^2_{g_{jk}};\ \mathrm{cov}(u_{ijk}, u_{imk}) = \sigma_{g_{jk,mk}};\ \mathrm{cov}\left(u_{ijk}, u_{ijo}\right) = 0 \quad (3)$$

$$var(b_{jl}) = \sigma^2_{b_j};\ var(e_{ijkl}) = \sigma^2_{e_j} \quad (4)$$

All random effects were assumed to be normally distributed and independent, unless stated otherwise. The general model consisted of a heterogeneous genetic variance structure for each species × experiment combination (for species other than unplanted). For the wheat (*T. aestivum*) species, it was assumed that $\text{cov}(u_{ijk}, u_{imk}) = 0$ due to the low number of wheat cultivars present within each experiment. For the purposes of the analysis, the wild *Cicer* derivatives from crossing with *C. arietinum* cultivars were included under the *C. arietinum* species.

To simplify the general model, a second variance structure was considered, allowing for modelling of the G × E interaction effects on a "crop type" basis, where crop types are defined by grouping together species belonging to the same genus (*Cicer*, *Triticum* and unplanted). This model enabled the estimation of heterogeneous genetic variance for each crop type × experiment combination. In both instances, independence was assumed between the species or crop types, and between experiments within the respective species or crop types. The variance structures were compared using a residual maximum likelihood ratio test (REMLRT) to inform which modelling approach offered the most parsimonious solution for modelling the G × E interaction, for each set of experiments. Once the most parsimonious solution for modelling the G × E interaction at the species or crop type level was determined, the G × E variance structures were extended to enable the modelling of genetic covariance between experiments at a species or crop type level.

Wald tests with an approximate *F* statistic were used to assess statistical significance of the fixed effects. When terms were significant as per the Wald test, Fisher's least significant difference (LSD) test was then used to perform multiple comparisons between treatment (e.g., species) levels. Estimates of the fixed effects were empirical best linear unbiased estimators (eBLUEs). Estimates of the random genotype effects for each experiment were empirical best linear unbiased predictors (eBLUPs). Spatial trends within each glasshouse experiment were adjusted for using the methods proposed in Gilmour et. al (1997). Back-transformed standard errors of the eBLUEs and eBLUPs were calculated using the delta method.

When assessing whether a particular accession had a significantly lower *P. neglectus* population density than a reference cultivar, a one-sided *Z* test was performed such that

$$Z = \frac{u_{ijk} - u_{\text{check } jo}}{sed_{ijk,\text{ check } jo}} \quad (5)$$

where u_{ijk} is the eBLUP for accession i in experiment j, $u_{\text{check } jo}$ is the eBLUP for the reference cultivar in experiment j, and $sed_{ijk,\text{ check } jo}$ is the standard error of difference between accession i and the reference cultivar in experiment j. If the genetic correlation between the two experiments being analysed was sufficiently large, then the eBLUPs were averaged across the set of two experiments prior to performing the one-sided *Z* test.

Provisional resistance ratings were given to all genotypes tested in both sets of experiments using the method described by Thompson et al. (2020). The range of $\log_e$ (*P. neglectus*/kg) as eBLUPs from the lowest genotype to the highest genotype (wheat cv. Gregory rated as S in consensus ratings) was subdivided into seven equal subranges. Genotypes within these subranges were assigned into seven ordinal categories as follows: resistant (R), resistant-moderately resistant (R-MR), moderately resistant (MR), moderately resistant-moderately susceptible (MR-MS), moderately susceptible (MS), moderately susceptible-susceptible (MS-S) and susceptible (S).

Practically, and as described previously, the accessions tested in Experiments 1 and 2 originated from the 2013 collection, while the accessions tested in Experiments 3 and 4 consisted of those obtained from both the 2013 and 2014 collection and a further array of *C. arietinum* breeding lines of interest. Furthermore, the two sets of experiments were conducted in different facilities, and although every caution was taken to ensure consistent experimental conduct, unmeasured and unforeseeable differences between the facilities could be expected. From a statistical perspective, exploratory data analysis identified that if all experiments were combined into a single analysis, it would be difficult to determine if the significant species × experiment interaction was due to differences in the genotypes present within each experiment as opposed to differences in genotypic performance across experiments. To avoid ambiguity in the interpretation of a significant species × experiment interaction, and because the primary focus was on differences in response to *P. neglectus* at the genotype level, a separate multi-experiment analysis was performed for (a) Set 1 (Experiments 1 and 2) and (b) Set 2 (Experiments 3 and 4).

All hypothesis testing was completed at the 5% significance level and all models were fitted using the ASReml-R package in the R software environment, which implements residual maximum likelihood (REML; Patterson & Thompson, 1971) estimation. All *P. neglectus* population densities discussed in the following results are back-transformed means presented as *P. neglectus*/kg.

3 | RESULTS

3.1 | Set 1 – Multi-experiment analysis of *P. neglectus* population densities

The multi-experiment analysis of Experiments 1 and 2 found that the species × experiment interaction was not statistically significant ($p = 0.19$). There was a significant species main effect ($p < 0.001$) with the unplanted treatment having significantly lower *P. neglectus*/kg than all *Cicer* species. There was no significant difference in the *P. neglectus* response level on average between *C. echinospermum* (6565 *P. neglectus*/kg), *C. reticulatum* (7790 *P. neglectus*/kg) and cultivated chickpea *C. arietinum* (8128 *P. neglectus*/kg) when an LSD test was performed (Table 4).

At the genotype level, the REMLRT indicated there was no significant improvement in the model when fitting heterogeneous genetic variance for each chickpea species within each experiment. Thus, homogeneous genetic variance was fitted across all chickpea species within an experiment. The genetic variance for wild *Cicer*

accessions and cultivated chickpea was statistically significant for both Experiments 1 ($p < 0.001$) and 2 ($p < 0.001$), indicating that within each experiment there was variation in response levels to *P. neglectus* between chickpea species.

There was strong genetic correlation between Experiments 1 and 2 ($\rho = 0.84$, Figure 2). Thus, predictions for chickpea genotypes were averaged across the two experiments (Figure 3, Table S1). Wheat reference cultivars, used to confirm multiplication of *P. neglectus*, performed as expected (population densities shown in Table S1). The lowest *P. neglectus* population densities were in the unplanted treatment (1591 *P. neglectus*/kg). *P. neglectus* population densities for *C. reticulatum* ranged from 3467 *P. neglectus*/kg for accession Oyali_073 to 14,138 *P. neglectus*/kg for accession Kalka_074, while population densities for *C. echinospermum* ranged from 4598 *P. neglectus*/kg for accession Karab_082 to 12,321 *P. neglectus*/kg for accession S2Drd_062. The lowest *P. neglectus* population densities of Australian commercial chickpea cultivars tested was PBA Pistol (6010 *P. neglectus*/kg) and PBA HatTrick (6494 *P. neglectus*/kg). Of the Australian chickpea breeding lines tested, D05253>F3TMWR2AB001 had the lowest *P. neglectus* population densities of 5639 *P. neglectus*/kg. D05253>F3TMWR2AB001 was derived from crosses with a *C. reticulatum* accession from the original genebank collection, ILWC 140 (6921 *P. neglectus*/kg) and the Australian commercial chickpea cv. Howzat (7742 *P. neglectus*/kg).

One *C. reticulatum* accession, Oyali_073, produced significantly ($p > 0.05$) lower *P. neglectus* population densities than Australia's elite chickpea cultivar and breeding parent PBA HatTrick (Table S1). Two *C. reticulatum* accessions, Oyali_073 and CudiB_008B, produced lower (but nonsignificant, $p > 0.05$) *P. neglectus* population densities than the Australian breeding line D05253>F3TMWR2AB001. Using the method described by Thompson et al. (2020), provisional resistance ratings were given to all wild *Cicer* accessions tested. Twenty-four wild *Cicer* accessions (19 *C. reticulatum* and 5 *C. echinospermum*) were given an R rating. The eBLUPs and provisional resistance ratings for *P. neglectus* population densities for all accessions evaluated in Experiments 1 and 2 are given in Table S1.

Wild *Cicer* accessions tested in Experiments 1 and 2 originated from 21 collection sites within five provinces of Turkey. The number of accessions varied between collection sites and within a collection site there was a range of *P. neglectus* population densities. This is shown using violin plots (Figure 4a,b) that were generated using the back-transformed mean of accessions (Table S1).

TABLE 4 Empirical best linear unbiased estimates of *Pratylenchus neglectus*/kg after 16 weeks' growth for the *Cicer* species main effect in multi-experiment analysis of Experiments 1 and 2

		P. neglectus/kg over-dried soil	
Species	n[a]	Log_e	Back-transformed mean
C. arietinum	18	9.00 a	8128
C. reticulatum	133	8.96 a	7790
C. echinospermum	41	8.79 a	6565

Note: There was no significant species-by-experiment interaction and thus *Cicer* species predictions were averaged across Experiments 1 and 2. Species with a letter in common are not significantly different as per an LSD test at the 5% level.

[a]*n*, number of accessions.

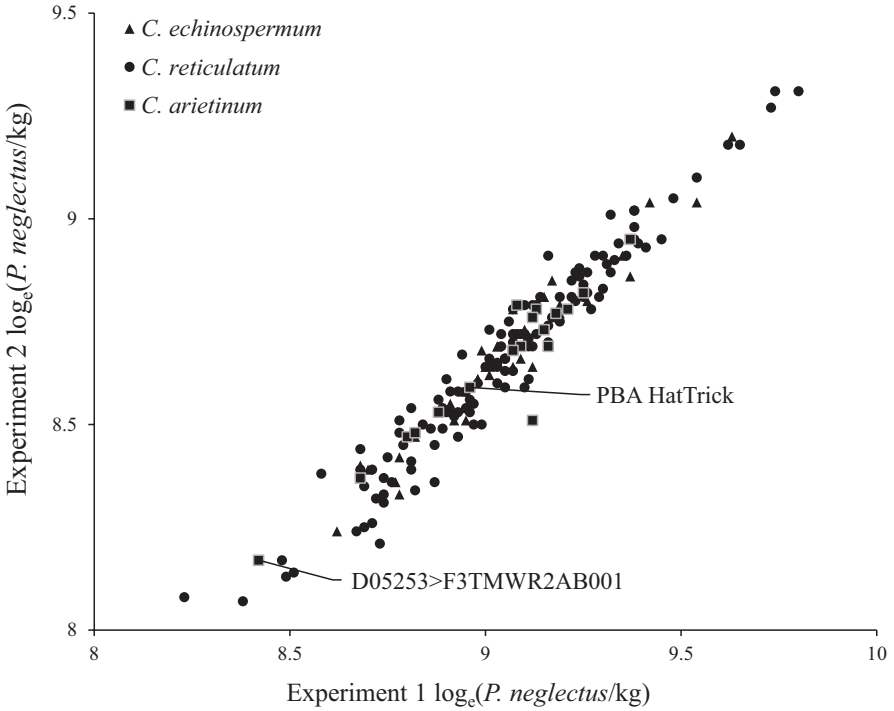

FIGURE 2 Empirical best linear unbiased predictions of *Pratylenchus neglectus* population densities for *Cicer* accessions calculated separately for each genotype from Experiments 1 and 2. The genetic correlation between the two experiments was strong ($\rho = 0.84$). PBA HatTrick is the elite chickpea cultivar chosen to represent Australia for the nested association mapping population produced from the wild *Cicer* collection and D05253>F3TMWRAB001 is Australia's current best breeding line with wild *Cicer* derivatives for *P. neglectus* resistance. *P. neglectus*/kg is based on extraction from soil and roots

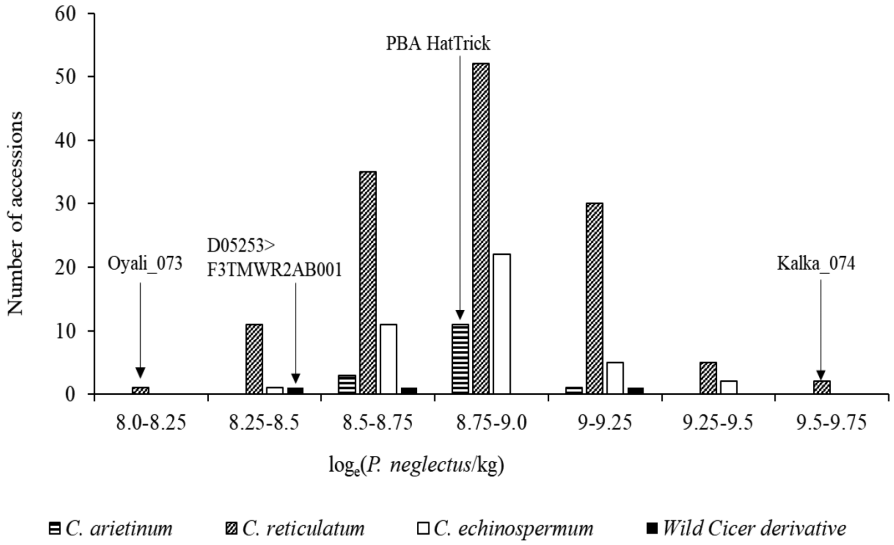

FIGURE 3 Frequency distributions of empirical best linear unbiased predictions of *Pratylenchus neglectus* population densities for cultivated chickpea and wild *Cicer* accessions after 16 weeks' growth averaged across Experiments 1 and 2. Genotypes listed are the wild *Cicer* that had both the lowest and highest *P. neglectus*/kg. PBA HatTrick is the elite chickpea cultivar chosen to represent Australia for the nested association mapping population produced from the wild *Cicer* collection and D05253>F3TMWRAB001 is Australia's current best breeding line with wild *Cicer* derivatives for *P. neglectus* resistance. *P. neglectus*/kg is based on extraction from soil and roots

3.2 | Set 2 – Multi-experiment analysis of *P. neglectus* population densities

Comparison of the species tested in Experiments 3 and 4 found that the species × experiment interaction was nonsignificant ($p = 0.11$). The species main effect between wild *Cicer* and cultivated chickpea *C. arietinum* was statistically significant ($p < 0.001$) with the LSD test, indicating that on average there was significantly lower *P. neglectus* population densities in wild *Cicer*, *C. echinospermum* (3413 *P. neglectus*/kg) and *C. reticulatum* (4627 *P. neglectus*/kg) compared to cultivated chickpea *C. arietinum* (7301 *P. neglectus*/kg) (Table 5).

Similar to the multi-experiment analysis for Set 1, a homogeneous genetic variance was fitted across all *Cicer* species as there was no evidence of genetic variance heterogeneity across the *Cicer* species as per the REMLRT. The genetic variance for the chickpea genotypes was also significant for Experiments 3 ($p < 0.001$) and 4 ($p < 0.001$) as per the REMLRT.

The genetic correlation between Experiments 3 and 4 was moderate ($\rho = 0.58$, Figure 5) indicating that a small to moderate amount of genotype × environment interaction may be present between Experiments 3 and 4. The genetic correlation was deemed strong enough to justify averaging predictions across Experiments 3 and 4, which is presented in Figure 6. Wheat reference cultivars, used to confirm multiplication of *P. neglectus*, performed as expected (population densities shown in Table S2).

The lowest *P. neglectus* population density was in the unplanted treatment (975 *P. neglectus*/kg). Values of *P. neglectus* population densities for *C. reticulatum* ranged from 1945 *P. neglectus*/kg for accession Bari3_106D to 8170 *P. neglectus*/kg for accession Dogan_033, while population densities for *C. echinospermum* ranged from 2260 *P. neglectus*/kg for accession Isoha_025 to 7090 *P. neglectus*/kg for accession Isoha_049. The lowest *P. neglectus* population density of Australian commercial chickpea cultivars tested was Moti at 2492 *P. neglectus*/kg. A total of 34 wild *Cicer* accessions had significantly ($p < 0.05$) lower *P. neglectus* population densities than Australia's elite breeding cultivar PBA HatTrick at 5628 *P. neglectus*/kg. Of the Australian chickpea breeding lines tested, 00283–1095–1002 (2461 *P. neglectus*/kg) and D05253>F3TMWR2AB001 (3152 *P. neglectus*/kg) had the lowest *P. neglectus* population densities, both derived from crosses with the *C. reticulatum* accession, ILWC 140 (3224 *P. neglectus*/kg). The Australian commercial chickpea cultivars used to make these crosses were Jimbour (3163 *P. neglectus*/kg) for 00283–1095–1002 and Howzat (3099 *P. neglectus*/kg) for D05253>F3TMWR2AB001. Fourteen wild *Cicer* accessions (10 *C. reticulatum* and 4 *C. echinospermum*) were given a provisional R rating. The eBLUPs and provisional resistance ratings for *P. neglectus* population densities for all accessions evaluated in Experiments 3 and 4 are given in Table S2.

Wild *Cicer* accessions tested in Experiments 3 and 4 originated from 26 and 32 collection sites, respectively, within seven provinces of Turkey. Like Experiments 1 and 2, the number of accessions varied between collection sites, and there was a range of *P. neglectus* population densities for accessions within collection sites for Experiments 3 and 4 as indicated by the violin plots (Figure 7a,b) using the back-transformed mean of accessions (Table S2).

3.3 | Nested association mapping parents

Analysis of the *P. neglectus* population densities for the 26 diverse wild *Cicer* NAM parent accessions from Experiments 3 and 4 revealed that 25 of the 26 produced lower population densities than

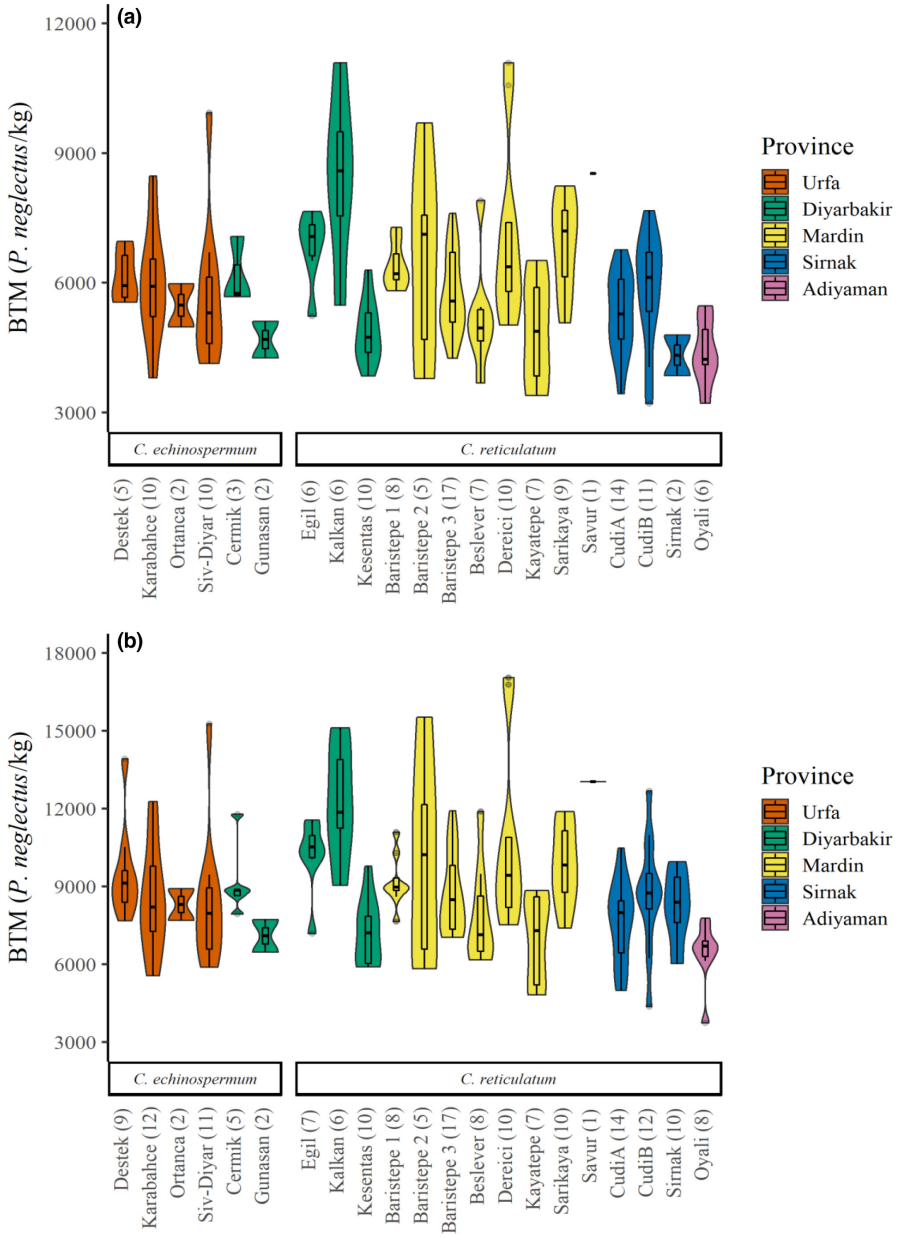

FIGURE 4 Violin plots with embedded boxplots showing the distribution of back-transformed mean *Pratylenchus neglectus* population densities for 21 wild *Cicer* collection sites in (a) Experiment 1 and (b) Experiment 2. The numbers of accessions from each collection site are shown in parentheses. *P. neglectus*/kg is based on extraction from soil and roots

TABLE 5 Empirical best linear unbiased estimates of *Pratylenchus neglectus*/kg after 16 weeks' growth for *Cicer* species main effect in multi-experiment analysis of Experiments 3 and 4.

		P. neglectus/kg oven-dried soil	
Species	***n*[a]**	**Log_e**	**Back-transformed mean**
C. arietinum	29	8.90 a	7301
C. reticulatum	146	8.44 b	4627
C. echinospermum	56	8.14 c	3413

Note: There was no significant species by experiment interaction and thus *Cicer* species predictions were averaged across Experiments 3 and 4. Species without a letter in common are significantly different as per an LSD test at the 5% level.

[a]*n*, number of accessions.

the breeding parent PBA HatTrick (Figure 8). Of these 25 accessions, four accessions, Bari3_106D (1945 *P. neglectus*/kg), Kayat_077 (2536 *P. neglectus*/kg), Oyali_084 (2976 *P. neglectus*/kg) and CudiB_022C (3020 *P. neglectus*/kg) produced significantly ($p < 0.05$) lower *P. neglectus* population densities than PBA HatTrick (Figure 8). Of the NAM parents, two accessions (Bari3_106D and Kayat_077) were rated R and seven accessions (Oyali_084, CudiB_022C, Derei_070, Besev_079, Bari2_072, Bari3_100 and Bari1_092) rated R-MR.

4 | DISCUSSION

This is the first known report on *P. neglectus* response in the new wild *Cicer* collection, assessing *C. reticulatum* and *C. echinospermum* accessions from both the 2013 and 2014 collection missions.

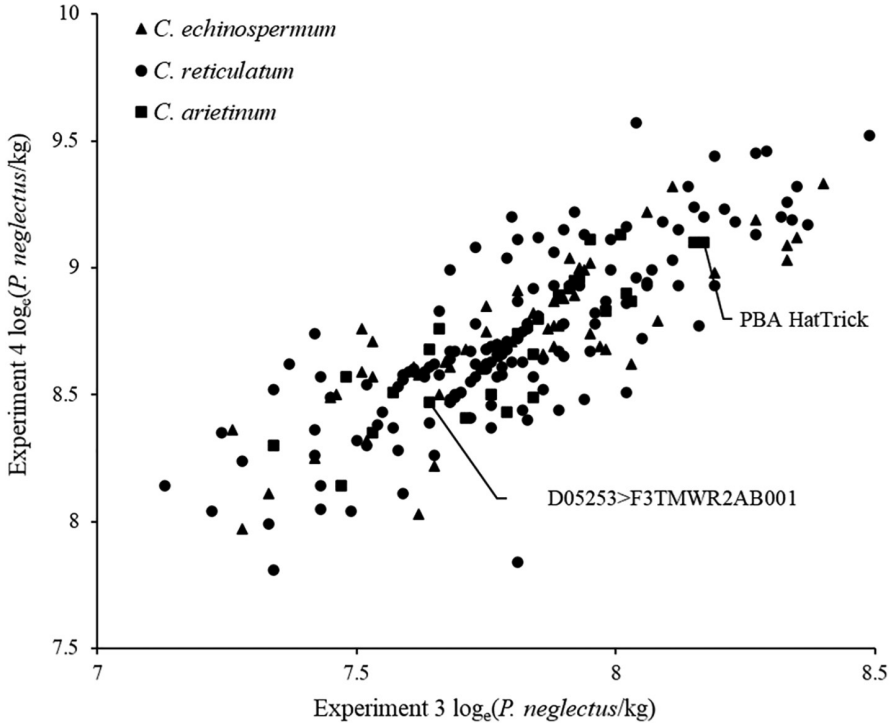

FIGURE 5 Empirical best linear unbiased predictions of *Pratylenchus neglectus* population densities for *Cicer* accessions calculated separately for each genotype from Experiments 3 and 4. The genetic correlation between the two experiments was moderate (ρ = 0.58). PBA HatTrick is the elite chickpea cultivar chosen to represent Australia for the nested association mapping population produced from the wild *Cicer* collection and D05253>F3TMWRAB001 is Australia's current best breeding line with wild *Cicer* derivatives for *P. neglectus* resistance. *P. neglectus*/kg is based on extraction from soil and roots

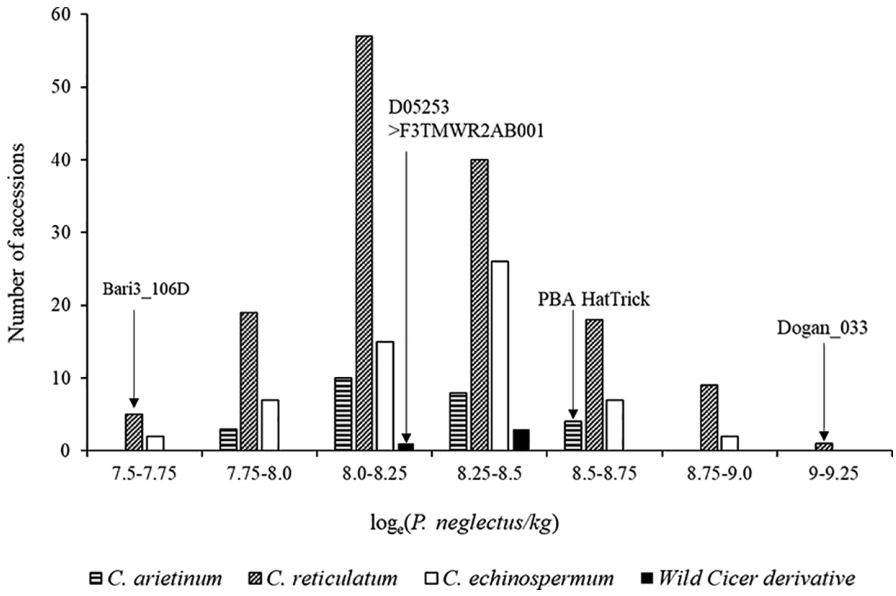

FIGURE 6 Frequency distributions of empirical best linear unbiased predictions of *Pratylenchus neglectus* population densities for cultivated chickpea and wild *Cicer* accessions after 16 weeks' growth averaged across Experiments 3 and 4. Genotypes listed are the wild *Cicer* that had both the lowest and highest *P. neglectus*/kg. PBA HatTrick is the elite chickpea cultivar chosen to represent Australia for the nested association mapping population produced from the wild *Cicer* collection and D05253>F3TMWRAB001 is Australia's current best breeding line with wild *Cicer* derivatives for *P. neglectus* resistance. *P. neglectus*/kg is based on extraction from soil and roots

This study has identified several wild *Cicer* accessions with better resistance to *P. neglectus* that can be used in the future breeding programmes and for targeted genetic exploration. Furthermore, the data provide newfound information in terms of *P. neglectus* resistance by characterizing 329 of the 590 wild *Cicer* accessions acquired from south-east Turkey for the new collection (Toker et al., 2021). PBA HatTrick is used as a benchmark for *P. neglectus* response in this study as it is the elite Australian chickpea breeding parent for crossing and genetic studies for this wild *Cicer* collection. The results of this study showed that only one accession had significantly lower *P. neglectus* population densities than PBA HatTrick in Experiments 1 and 2, while 34 wild accessions had significantly lower *P. neglectus* population densities than PBA HatTrick in Experiments 3 and 4. Twenty of these were new accessions received in Set 2 in 2018,

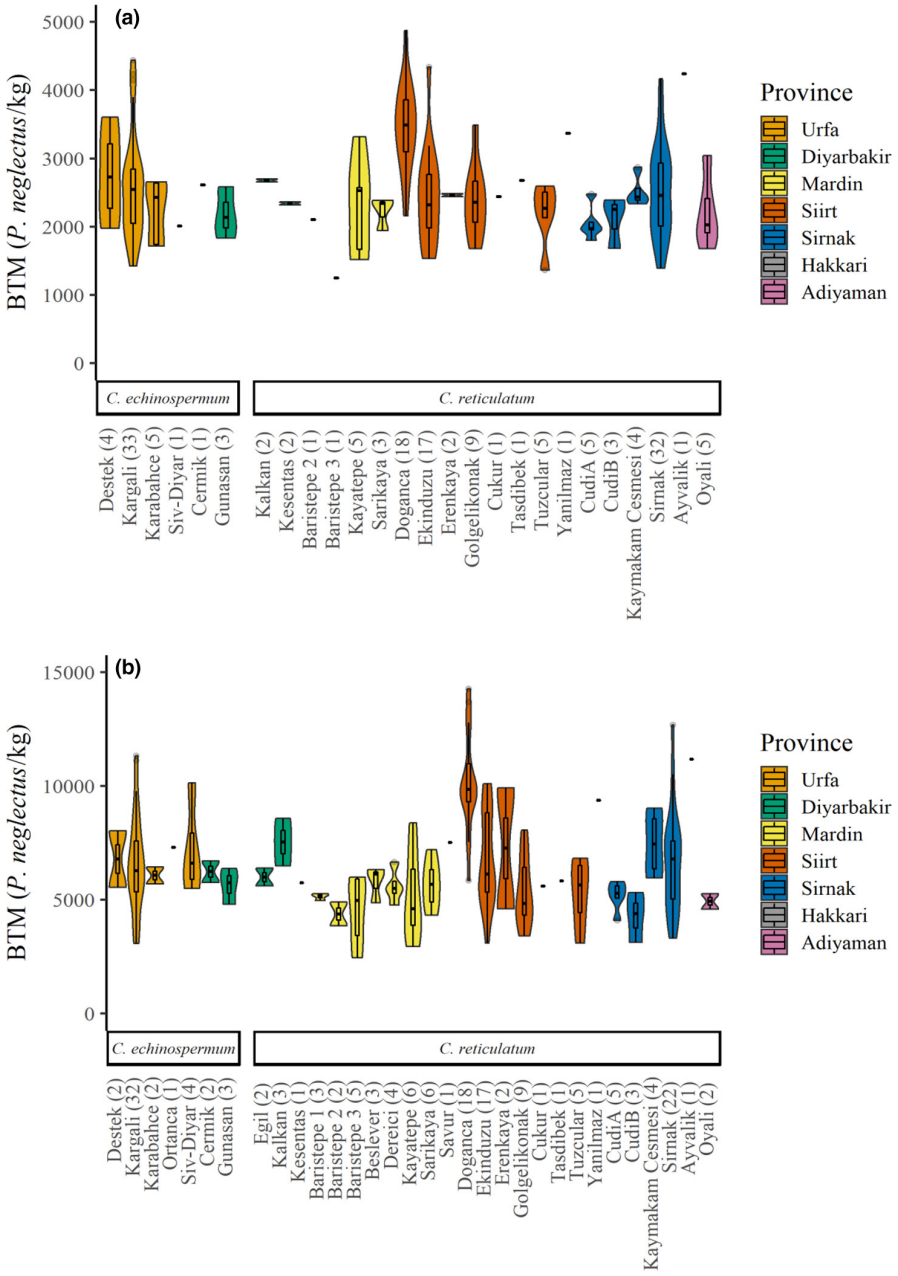

FIGURE 7 Violin plots with embedded boxplots showing the distribution of back-transformed mean *Pratylenchus neglectus* population densities for (a) 26 wild *Cicer* collection sites in Experiment 3 and (b) 32 wild *Cicer* collection sites in Experiment 4. The numbers of accessions from each collection site are shown in parentheses. *P. neglectus*/kg is based on extraction from soil and roots

demonstrating that expanding on and phenotyping accessions from different collection sites identified an increased number of accessions with improved resistance responses to *P. neglectus* and prevented this collection from being under-represented.

An earlier study by Thompson et al. (2011) also showed greater *P. neglectus* resistance in a small number of original wild *Cicer* that included nine *C. reticulatum* and five *C. echinospermum* from the original genebank collection. This current study has identified a range of *P. neglectus* responses in *C. reticulatum* and *C. echinospermum* and from the larger sample size has better represented the resistance status of both wild *Cicer* species compared to the Thompson et al. (2011) study. Thompson et al. (2011) highlighted ILWC 140 as an accession with greater resistance to *P. neglectus* than *C. arietinum*. It was then used in plant breeding as a parent to create many of the breeding lines tested in this study. In this current study, accessions that produced significantly lower *P. neglectus* population densities than known resistant to moderately resistant Australian chickpea cultivars predominately consisted of *C. reticulatum* accessions. This is beneficial, as *C. reticulatum* is directly cross-compatible with *C. arietinum* making it a more desirable choice for breeders to use in breeding programmes. Although *C. echinospermum* can be crossed with *C. arietinum*, there is greater chance of sterility and loss of vital genetic material (Croser et al., 2003).

To date, there is no published literature on chromosomal regions in chickpea associated with *P. neglectus* resistance. Channale et al. (2021) found that numerous genes participated in defence pathways to provide resistance against *P. thornei* in chickpea. This suggests that *P. thornei* resistance in chickpea is polygenic (Channale et al., 2021). It is also hypothesized that *P. neglectus* resistance in chickpea is polygenic, although no genetic studies have yet been performed

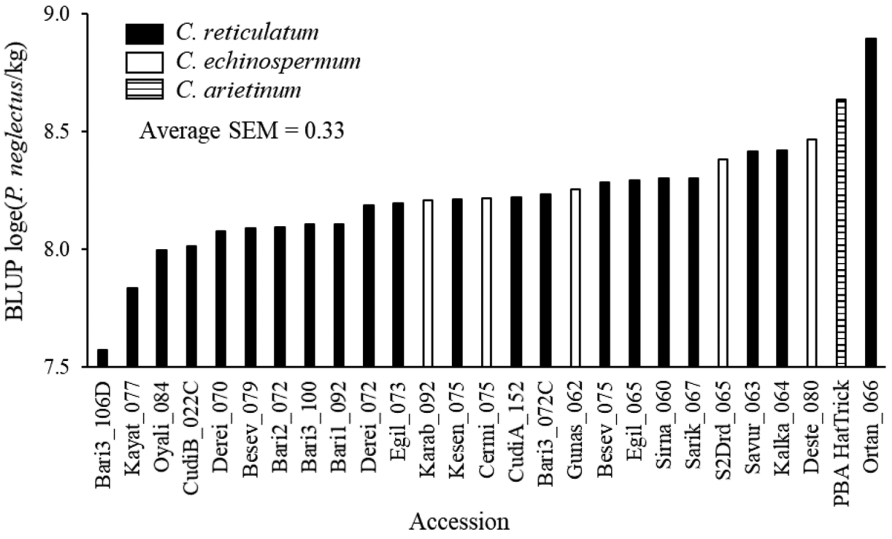

FIGURE 8 *Pratylenchus neglectus* empirical best linear unbiased predictions for the 26 nested association mapping wild *Cicer* parents (*Cicer reticulatum* shown in black; *C. echinospermum* shown in white) and the common parent PBA HatTrick (hashed) from multi-experiment analysis of Experiments 3 and 4. *SEM*, standard error of the mean. *P. neglectus*/kg is based on extraction from soil and roots

with *P. neglectus*. In this study the Australian wild relative-derived breeding line D05253>F3TMWR2AB001 performed better than its parent ILWC 140 for *P. neglectus* resistance. A future genome-wide association study will be important to identify if resistance in the wild *Cicer* accessions tested in this study differ in genomic regions compared with earlier accessions from the original genebank collection, such as ILWC 140. This information will also inform if greater genetic variance and resistance is already available in D05253>F3TMWR2AB001 or if there are novel sources of resistance that could be used from the new wild *Cicer* accessions for future breeding. Molecular markers derived from candidate genes for resistance will be useful tools for marker-assisted selection to incorporate new sources of superior resistance into chickpea breeding programmes.

In Australia and worldwide, greater resistance to multiple abiotic and biotic constraints in chickpea conveys maximum yield and profitability to the crop. Root-lesion nematode *P. thornei*, pod borer and Ascochyta blight are three major biotic constraints to the chickpea industry worldwide. Under conducive disease/pest conditions, recorded yield losses in chickpea have been 20%–50% from *P. thornei* (Reen et al., 2014; Di Vito et al., 1992), 80%–90% from pod borer (Sehgal & Ujagir, 1990, p. 30) and up to 100% from Ascochyta blight (Nene & Reddy, 1987). Using data from previous studies published on the new wild *Cicer* collection, along with information from this study, multiple resistance has been identified in several of the wild *Cicer* accessions from the 2013 collection. Six *C. reticulatum* accessions that produced lower *P. neglectus* population densities than PBA HatTrick in both sets of experiments (Bari2_062, Bari3_106D, Kayat_061, Kayat_066, Oyali_073 and Oyali_084) also produced significantly ($p < 0.05$) lower *P. thornei* population densities than PBA HatTrick (Reen et al., 2019). The *C. echinospermum* NAM parent Gunas_062 produced lower *P. neglectus* population densities than PBA HatTrick in this study and also for *P. thornei* (Reen et al., 2019) and pod borer (von Wettberg et al., 2018), and was highly resistant to stem damage from a mixture of Ascochyta blight strains (Newman et al., 2021). The *C. reticulatum* accession CudiB_008B produced one of the lowest *P. neglectus* population densities in this study and was also highly resistant to an Ascochyta blight mixture on the stem area and tolerant for the leaf area (Newman et al., 2021). However, it should be noted that unlike this current study, previously published studies on the new collection were focused only on the 2013 collection mission. These included the 26 NAM parent accessions tested for pod borer resistance (20 *C. reticulatum* and 6 *C. echinospermum*) by von Wettberg et al. (2018), 133 *C. reticulatum* and 41 *C. echinospermum* for *P. thornei* resistance by Reen et al. (2019), and 149 *C. reticulatum* and 48 *C. echinospermum* for Ascochyta blight resistance by Newman et al. (2021).

Accessions tested in this study were collected from 32 sites within seven provinces of south-east Turkey, which encompassed the full geographical range of the species (von Wettberg et al., 2018). von Wettberg et al. (2018) analysed site environments to determine elevation gradient, climatic and soil differences, which identified that *C. echinospermum* occurs at elevations of 740–1264 m a.s.l. whereas *C. reticulatum* occurs at generally higher elevations of 915–1695 m a.s.l. This explained some genetic variation between the species (von Wettberg et al., 2018). However, there was no trend between *P. neglectus* response of accessions and collection site as there was a range of *P. neglectus* population densities in collection sites that occurred at different geographical and elevational locations.

P. neglectus reduces root branching and limited studies have shown wild *Cicer* species also have smaller root systems than *C. arietinum* (Chen et al., 2016; Kashiwagi et al., 2005). This could imply low nematode population densities are the result of damaged or smaller root systems and not due to resistance. However, Reen et al. (2019) conducted correlation analyses between root biomass and final *P. thornei* population densities and found no significant relationship for *C. reticulatum*, *C. echinospermum* or *C. arietinum*. Similarly, the mean root biomass did not differ significantly among these *Cicer* species. Lesions on the roots of infected plants are indicative of *P. neglectus* infestation; however, measuring lesions of infected roots is not recommended for determining levels of resistance (Ali & Ahmad, 2000). This is due to the lesions being a

symptom of infestation, rather than an indication of actual *P. neglectus* numbers present in the roots and soil, which determines the resistance or susceptibility of a host. It is also important to note that initial planting inoculation density may not accurately reflect the number of *P. neglectus* that actually infects roots, with Vanstone and Nicol (1993) reporting that only 27%–37% of *P. neglectus* successfully penetrated roots in a glasshouse experiment using 300-ml pots. Therefore, to best categorize wild *Cicer* accessions for resistance response we have used the method of Thompson et al. (2020) to give a provisional resistance ranking to wild *Cicer* accessions and also provide probability scores of genotypes more resistant than PBA HatTrick at $p < 0.05$. Final nematode population densities for the same accession can vary between experiments (Kaplan, 1990), which also affects resistance ratings between experiments, resulting in slight variation in relative ratings.

This study has demonstrated the diversity of *P. neglectus* response to over 300 wild *Cicer* accessions that can be used in the future breeding programmes by identifying wild *Cicer* accessions with greater resistance than current Australian commercial chickpea cultivars. The current Australian breeding line D05253>F3TMWR2AB001 is also still a strong candidate for proceeding as there were no wild *Cicer* accessions that produced significantly lower *P. neglectus* population densities than this line. Through this study and the bringing together of information from previous studies, it is clear there are wild *Cicer* accessions that have possible multiple resistance to *P. neglectus*, *P. thornei*, Ascochyta blight and pod borer that would be highly advantageous in subsequent breeding programmes. These results can also be used globally in countries where *P. neglectus* is a constraint to chickpea production.

Wild *Cicer* are integral for genetic disease improvement in cultivated chickpea, with this research enabling breeders and industry to make informed decisions regarding *P. neglectus* resistance. The information provided here will support future development of more robust chickpea cultivars that will limit nematode reproduction in infested soils, resulting in greater yields and more profitable grains industries.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Hannah E. Rostad https://orcid.org/0000-0001-5647-1029
Roslyn A. Reen https://orcid.org/0000-0002-6875-3098
Michael H. Mumford https://orcid.org/0000-0002-5037-8680
Rebecca S. Zwart https://orcid.org/0000-0002-5634-7439
John P. Thompson https://orcid.org/0000-0002-2097-5695

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