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Information package to support Final Report – Biological control of fireweed (*Senecio madagascariensis*) Phase 3 (Grant Activity ID: 4-ENYS378)

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Stage 1 Host-specificity testing of the top-ranked priority candidate biocontrol agent, *Gasteroclisus tricostalis*

1 Establishment and maintenance of a lab-reared culture of *G. tricostalis* at the University of KwaZulu-Natal (UKZN) laboratories, South Africa, for importation to Australia

1.1 Fieldwork to collect *Gasteroclisus tricostalis* weevils in KwaZulu-Natal, South Africa

Between 1st January 2020 and 5th March 2023, a total of 83 field excursions were undertaken at 25 sites, at which a total of 150 healthy weevils were collected (**Table 1, Figure 1**; note that 44 weevils had been collected in early 2020, prior to formal commencement of the project in June 2020). Adult weevils were collected from the field either by beating mature host plants over a tray or with a sweepnet (**Figure 1**). Four of these sites (Ashburton, Emanzini, Mphophomeni, and Ukulinga) comprised healthy weevil populations and, as such, were visited multiple times in order to maintain a steady supply of weevils for replenishment of the lab-reared weevil culture at the University of KwaZulu-Natal, and consignment to Australian researchers at CSIRO in Canberra.

The number of weevils detected in the field declined significantly between milestone reporting years despite an increase in sampling effort over time ($R^2 = 0.29$, $F = 32.49$, $P < 0.0001$, **Figure 2**). For example, in 2020, three trips were made to Ashburton and 25 weevils were collected, while in 2021 and 2022 eight and nine trips were made and 18 and 22 weevils were collected, respectively. When COVID-19 travel restrictions were eased in 2022, an increased effort was made to locate additional sampling sites (i.e., 17 additional sites visited) consisting of healthy and abundant weevil populations. Unfortunately, no weevils were detected at most of these additional sites.

1.2 Maintenance of the laboratory-reared culture of *G. tricostalis* at the University of KwaZulu-Natal, South Africa

Rearing methods

Field collected weevils were brought back to the laboratory and placed in large glass Petri dishes (15 cm diameter) for mating (**Figure 3a,b**). Weevils that paired off were removed from the Petri dish and confined to a single potted fireweed plant (30 cm – 50 cm height, > 0.5 cm stem diameter) surrounded by a 69 cm plastic sleeve for one week in the laboratory under LED plant growth lights (**Figure 3c**). Plants were propagated in 18 cm plastic pots containing potting soil and watered regularly, with temperature set at 24 °C, light at 12 hr day/12 hr night and ambient humidity. Approximately 1 tablespoon of honey was provided on the side of each plastic sleeve to supplement feeding by the adult weevils.

After one week, fireweed plants were removed from the laboratory and placed outdoors under the shade house at the UKZN botanical gardens where they were watered on an automatic timer. Plants were kept in the shade house for seven weeks, then returned to the laboratory where they were kept in BugDorm-2® insect tents (60 × 60 × 60 cm; MegaView Science; **Figure 3d**) for an additional four weeks under plant growth lights (**Figure 3d**). Plants were watered as required and the BugDorm cages were inspected regularly for emergence of adult weevils (i.e., F1 generation of lab-reared weevils).

Upon emergence, the F1 adult weevils were removed from the BugDorm cages and kept individually in small plastic Petri dishes (7 cm diameter) where they were fed fireweed shoot tips. Once several F1 weevils had emerged, they were all added to a large glass Petri dish for pairing. Any weevils that did not pair up were moved back to the smaller plastic Petri dishes.

After four weeks in the BugDorm cage, the fireweed plants were dissected to detect any larvae, pupae, or adults still in the stems that had not emerged. If larvae or pupae were found, the stems were resealed with parafilm and kept in a glass Petri dish until the adult weevils had emerged. Any dead larvae, pupae or adults were preserved in 1.5 ml Eppindorf tubes with 100 % ethanol for genetic analysis.

Status of the weevil culture in South Africa

Since the initiation of the culture in 2020, an average of 33 plants have been exposed to an average of 8 mating pairs of weevils every month. Altogether, a total of 115 adult weevils were reared at the UKZN laboratories over the course of the project. The number of weevils reared per year varied over time. In 2020, no weevils could be reared, in 2021 a total of 50 weevils were reared, in 2022 a total of 17 weevils were reared, and up until June 2023 a total of 48 weevils were reared.

We observed that the field-collected adult weevils (**Figure 4a**) retained at the UKZN laboratories were typically long-lived, with some individuals surviving for more than one year under laboratory conditions. We observed that female weevils have a pre-oviposition period of between 8 days and three weeks, during which time they must feed on healthy fireweed leaves (Matsunga et al. 2022). The number of eggs laid by each mating pair of weevils was highly variable, with some females laying fewer than 5 eggs per week whilst others laid over 20 per week on average.

We observed that female weevils chew a hole in the stems of fireweed and oviposit a single egg per hole, which is then closed with a faecal plug (**Figure 4b**). Eggs typically hatch after approximately 8 days (Matsunga et al. 2022), and larvae develop through 4 or 5 instars as they bore down the pith of the host fireweed plant (**Figure 4c**), weakening the stem (Matsunga et al. 2022). We observed that the duration of development from larval to adult emergence is highly variable and temperature dependant, with longer development times in winter, taking an average of 79 days (Matsunga et al. 2022).

During 2021, several attempts were made to transfer larvae to cut fireweed stems or whole plants, as some weevil pairs would lay 20 eggs in a single plant, yet typically no more than three adults could be reared from that same plant, which we speculate was a result of larval competition for food resources. Fireweed plants with more than six eggs were dissected after a week and any eggs and early instar larvae were transferred to cut fireweed stems. The stems were sealed with parafilm and kept in plastic Petri dishes. Larvae were transferred to fresh stems approximately twice per week. A total of 162 eggs and 119 larvae were transferred using this transfer method but only two larvae could be reared through to emergence of F1 adults. Since this larval transfer method is labour-intensive and had low success rate, it has since been discontinued.

Figure 1. Collecting weevils by (a) beating plants over a tray and (b) sweeping with a net.

Table 1. Details of field trips made to collect *G. tricostalis* weevils from 1 January 2020 to 24 March 2022 across KwaZulu-Natal, South Africa.

Site	GPS S; GPS E	Altitude (m amsl)	Date (year/month/day)	No. of weevils collected	Milestone reporting period[a]
Ashburton	29.6951S; 30.4955E	777	2020/11/13	10	1
			2020/11/30	5	1
			2020/12/11	10	1
			2021/09/15	1	2
			2021/10/08	4	2
			2021/10/13	1	2
			2021/10/15	4	2
			2021/10/20	2	2
			2021/11/03	0	2
			2021/11/10	6	2
			2021/12/15	0	2
			2022/02/03	0	2
			2022/02/17	0	2
			2022/03/10	1	2
			2022/03/24	4	2
			2022/03/31	8	2
			2022/09/08	0	3
			2022/10/13	5	3
			2022/10/30	2	3
			2022/12/20	2	3
			2023/01/20	0	3
			2023/02/22	2	3
			2023/03/22	1	3
Bishopstowe	29.5683S; 30.4501E	856	2022/09/22	0	3
Cato Ridge	29.5395S; 30.2684E	711	2021/10/01	0	2
Cedara	29.7723S; 30.6014E	711	2022/02/22	0	2
			2022/03/04	0	2
			2022/03/23	0	2
			2022/09/05	0	3
			2022/09/28	0	3
Curry's Post	Various		2022/10/06	0	3
Elysium	30.4834S; 30.6342E	11	2022/02/08	0	2
Emanzini	29.4638S; 30.3804E	658	2020/01/28	1	0
			2020/01/31	5	0
			2020/03/08	3	0
			2020/03/11	13	0
			2020/03/24	10	0
			2020/12/06	1	1
			2021/09/18	0	2
			2021/09/28	3	2
			2021/10/07	2	2
			2021/10/12	3	2
			2021/10/14	1	2
			2021/10/21	1	2
			2021/11/02	0	2

			2021/11/11	1	2
			2021/11/16	1	2
			2022/03/02	0	2
			2022/09/11	0	3
			2022/10/09	1	3
Fountain Hill	29.4475S; 30.5593E	873	2021/09/08	1	2
			2021/09/14	1	2
Greytown	29.0901S; 30.6000E	986	2022/03/03	0	2
Ground Cover	29.3886S; 30.1769E	1287	2023/02/23	0	3
Hammersdale 1	29.8113S; 30.6411E	698	2021/10/01	0	2
Hammersdale 2	29.8392S; 30.6881E	551	2021/10/01	0	2
Hluhluwe	Various		2022/10/10	0	3
Ifafa	30.4633S; 30.6522E	8	2022/02/08	1	2
Lions River	29.4662S; 30.1553E	1062	2022/02/24	0	2
Midlands 1	29.4836S; 30.9900E	1442	2021/09/21	0	2
Midlands 2	29.4561S; 29.9535E	1444	2021/09/21	0	2
Mooi River	29.2477S; 29.9923E	1418	2022/02/24	0	2
Mphopomeni	29.5469S; 30.1822E	1066	2020/01/01	3	0
			2020/02/02	9	0
			2022/10/29	2	3
Nottingham Road 1	29.3363S; 29.9938E	1447	2022/02/24	0	2
Nottingham Road 2	29.3947S; 29.9842S	1477	2023/03/02	0	3
Park Rynie	30.3262S; 30.7375E	12	2022/02/08	0	2
			2022/09/15	0	3
Tinley Manor	29.4520S; 31.2860E	11	2022/09/01	0	3
Ukulinga	29.6581S; 30.4073E	757	2022/02/16	0	2
			2022/02/23	1	2
			2022/03/01	3	2
			2022/03/07	3	2
			2022/03/22	4	2
			2022/03/30	4	2
			2022/09/29	3	3
			2022/10/14	2	3
			2022/10/30	3	3
			2023/03/09	0	3
			2023/03/29	0	3
			2023/05/05	0	3
Uvongo	30.8370S; 30.3956E	6	2022/09/15	0	3

[a] Note that “0” under the milestone reporting period column indicates weevils that were collected prior to formal commencement of this project.

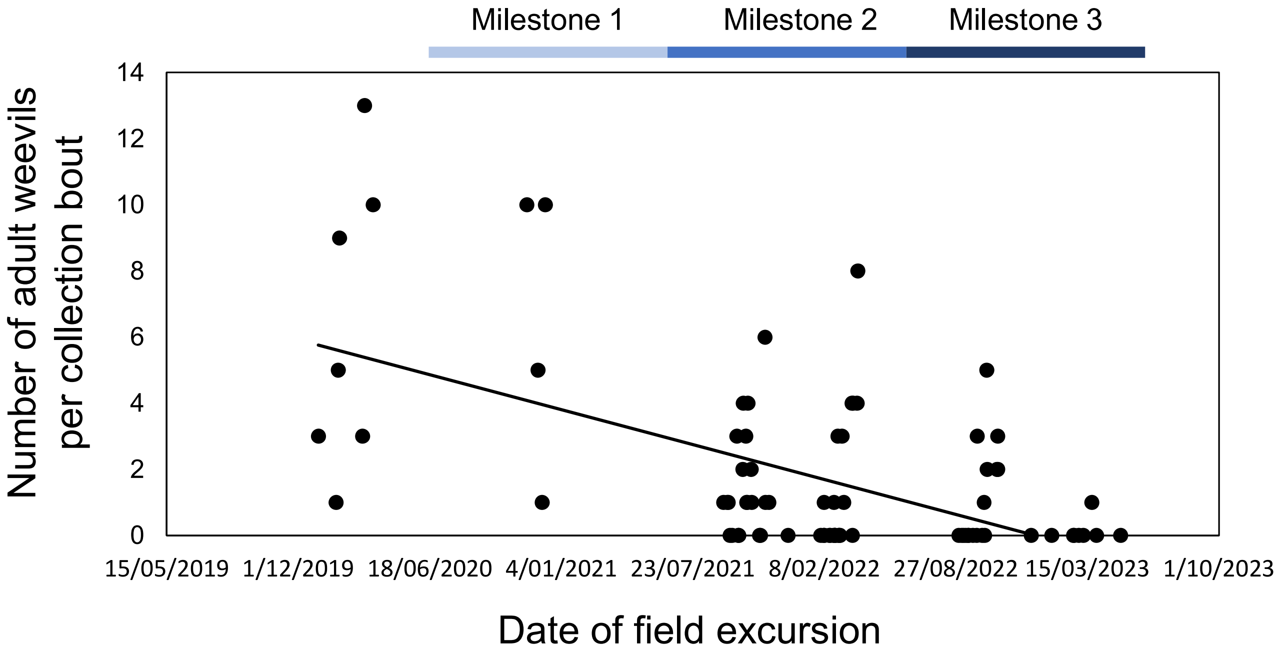


Figure 2. Variation in the number of *G. tricostalis* weevils collected per field trip over time (between January 2020 and March 2023). The black curve represents the line-of-best-fit based on regression analysis, demonstrating a significant ($P < 0.0001$) reduction in number of weevils collected during each sampling excursion. Coloured bars at the top of the graph denote the duration of each milestone reporting period.

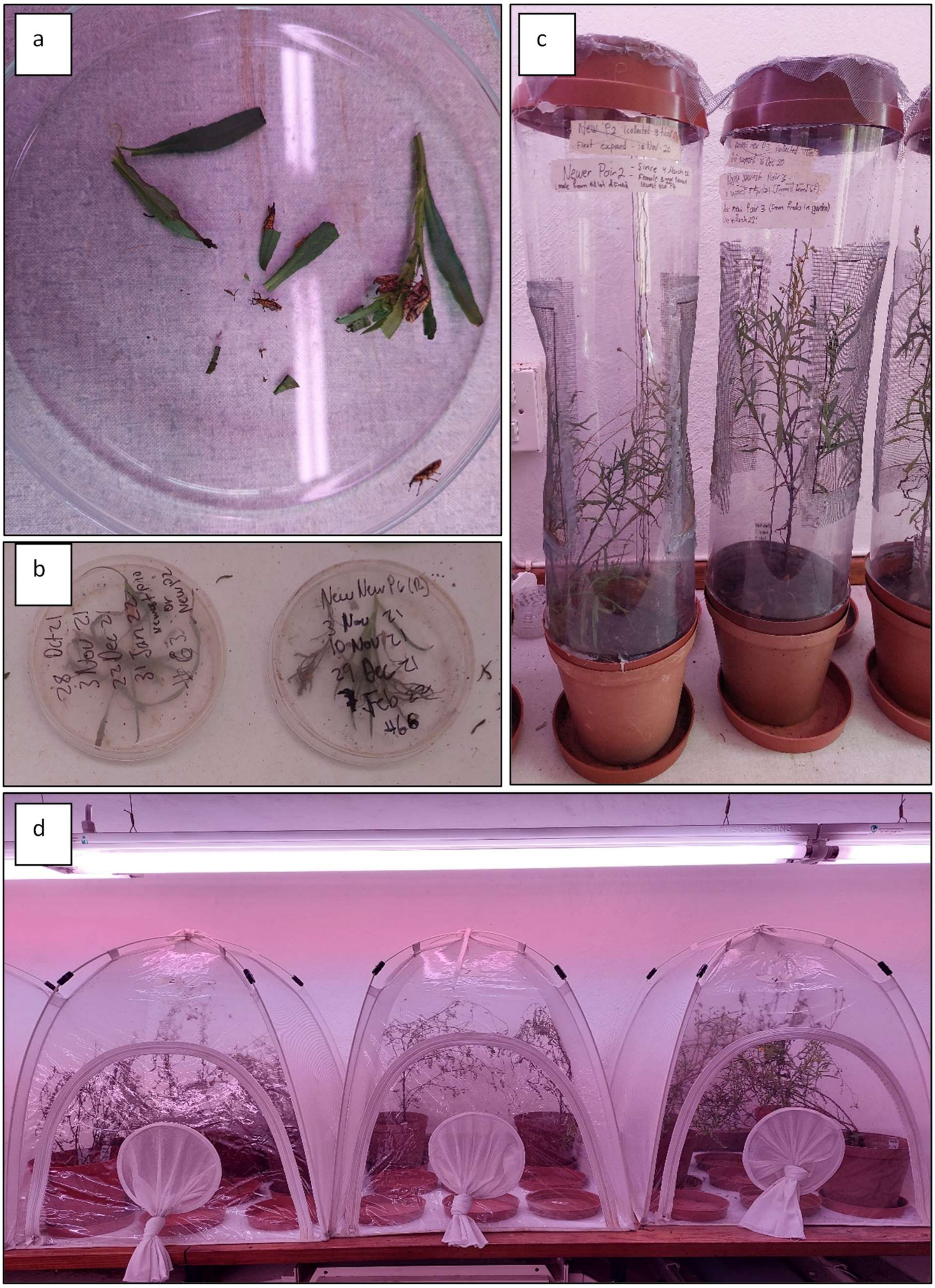


Figure 3. Weevil culturing: (a) large Petri dish for pairing adults, (b) small Petri dishes containing single adult weevils, (c) plastic sleeves with single potted fireweed plants and weevil pairs, and (d) BugDorm cages with multiple fireweed plants under grow lights.

Figure 4. Weevil reproduction: (a) two pairs of adult weevils, (b) oviposition holes (denoted by yellow arrows), and (c) larval stem-boring damage with frass.

2 Establishment of a healthy colony of *Gasteroclisus tricostalis* under quarantine conditions in Australia

2.1 Importation of field-collected weevils from South Africa to Australian quarantine facilities

In 2020 (milestone reporting year 1), a comprehensive package of information on the proposed biocontrol program (target weed, non-target native plants, biology of the candidate biocontrol agent) was prepared to support an application to the Australian Government regulators for importation and testing of the weevil under quarantine conditions at the CSIRO laboratories in Canberra (see **Appendix 12** for a copy of the information package, and **Table 2** for list of import and testing permit numbers).

Altogether, commencing in February 2021, six field-collected weevil colonies were consigned from KwaZulu-Natal in South African to CSIRO in Australia (details provided in **Table 2**, **Figure 5**). Due to travel restrictions as a result of the COVID-19 pandemic, most of these shipments were couriered via Europe and America rather than eastwards directly to Australia, greatly increasing travel time (up to two weeks) and resulting in up to 70 % mortality of the weevils upon arrival. We arranged for the two most recent colonies to be hand delivered to Australia, which took only two days and resulted in a 100 % survival of constituent weevils.

2.2 Colony establishment in Australian quarantine facilities

Context

The following section 2.2. is modulated into two parts: (1) preliminary investigations into the reproductive biology of the weevil in milestone years 1 and 2 (coinciding with the peak of COVID-19 associated travel restrictions), and (2) information on the successful establishment of a viable, thriving colony of the weevil in milestone year 3, resulting in population growth over multiple (F1 to F2) generations, as a foundation to deployment of weevils in host-specificity tests.

Part 1 – preliminary investigations into the reproductive biology of the weevil in milestone reporting years 1 and 2

<u>Survival of field-collected adult weevils under quarantine conditions</u>

More than 50 % of field-collected adult weevils were alive upon arrival in consignments 1 (BAA385917), 3 (AELHHYH79) and 5 (Q20276995) (**Table 2**). The daily survival rate (calculated as % of original number of adult weevils alive upon arrival) declined significantly through time, whereby all weevils from the first consignment had died by 150 days, whilst all weevils from the third consignment died by 50 days and the fifth consignment had died by 12 days (**Figure 6**). This result was surprising, given that the fifth consignment was carefully hand-delivered and took only 2 days to arrive in Australia from South Africa and thus we presume suffered less transit stress than the other two consignments. We observed that, between spring 2021-autumn 2022, the abundance of host fireweed plants and weevils in the field across South Africa were very low, so the fifth consignment of weevils may have been in a poor condition at the time of collection in the field. It is also possible that weevils collected in February 2021 and sent in the first consignment had only recently emerged from fireweed plants (and thus been less than a year old at the time of delivery), whilst those weevils included in the fifth consignment may have belonged to a cohort from the previous growing season and were thus a year or more old at the time of collection.

Patterns of oviposition of field-collected adult weevils on Australian accessions of fireweed

We carefully tracked patterns of oviposition of three cohorts of reproductively mature adult weevils in the Australian quarantine facilities over milestone reporting years 1 and 2: (1) a single breeding pair derived from the first consignment, (2) the remaining weevils from the first consignment and (3) all weevils from the third consignment (**Figure 7**). None of the 20 weevils from the fifth consignment were observed to pair up or lay any eggs on the host fireweed plants before they died several days after consignment.

There was remarkably high variability in fecundity (i.e., number of eggs laid per reproductively mature female) amongst weevils, with approximately two-thirds of all eggs being laid by the single long-lived female weevil derived from the first consignment that arrived in Australia in March 2021 (dark-blue curve in **Figure 7**). The time until first egg laying also varied considerably, with the single female from the first consignment commencing oviposition within 20 days (and maximum oviposition at ~ 100 days), whilst the remaining female weevils from the first consignment only commenced oviposition after 60 days. The reasons for such high variability in fecundity amongst individual weevils and cohorts remain unknown but may relate to the health of the female at the time of exposure to the Australian accessions of the fireweed plants or variation in the genotypic compatibility amongst male and female weevil pairs.

Larval development through to pupation and emergence of F1 adults

We carefully tagged and tracked individual oviposition holes on fireweed stems over ~140 days to evaluate rates of larval development through to pupation and emergence of F1 adult weevils (**Table 3**). We also tracked development rates over two ambient temperatures (17 vs 22 °C) to further optimise culturing conditions within the quarantine facility.

Of the 25 oviposition holes (in which we assumed that a single viable, fertilised egg was laid), 21 larvae (84 %) developed through to late instar (pre-pupation) stage (on average by 56 days, **Table 3**). The average number of days to pupation was 88 days. Subsequently, 18 (72 %) F1 adults emerged from pupae (note that 2 adults died whilst emerging from pupae, thus only 16 F1 adults were reared altogether, **Table 3**). Unfortunately, all 16 of these F1 adults subsequently died by 28 days post emergence (average ± SD number of days until death = 16.75 ± 8.00). On average, the number of days until emergence of F1 adult weevils reared at 17 °C was 30 % higher (~119 days) than those reared at 22 °C (~93 days), thus providing evidence of temperature-dependent development in this species.

We also observed the development of gall-like structures (i.e., distinctly thickened swellings at the junctions of main and lateral fireweed stems), where late instar larvae pupated in 9 (36 %) of 25 cases (**Figure 8**). These observations were unexpected as galls have never been reported by researchers working on lab-reared colonies of *G. tricostalis* in Hawaii or South Africa and not been observed on fireweed plants in the field in South Africa. In all instances, once these swollen structures had formed, the larvae never tunnelled further beyond that point down the fireweed stem. The mechanisms underpinning gall formation (e.g., possible plant defence response) should be investigated, as this may point to some intrinsic differences (possibly genotypic) in how the weevil interacts with its host plant between native and introduced fireweed populations, with implications for the efficacy of the weevil if introduced into the Australian environment.

Part 2 – successful establishment of a viable weevil colony in milestone year 3, as a basis for host-specificity trials

Survival of field-collected adult weevils under quarantine conditions

The percentage survival of the most recent consignment of weevils (consignment number NAA22059912) declined linearly upon arrival at the CSIRO laboratories in Canberra up until approximately 80 days, after which a subset of 25 % of the field-collected weevils survived until 2nd May 2023 (red curve in **Figure 6**), and only three adults remaining by 2nd June 2023.

Patterns of oviposition of field-collected adult weevils and development of F1 generation on Australian accessions of fireweed

Upon receipt, weevils were confined to cages containing healthy and reproductively mature fireweed plants (Bega Valley accession) and monitored every couple of days for adult feeding on fireweed foliage, male and female pairing, and oviposition. Adults that paired up were then moved to separate cages containing fresh fireweed plants. Each oviposition hole was individually tagged and monitored over time. Altogether, this weevil cohort laid 344 eggs (dated to 2nd March 2023; NB – given the rapidly growing size of the culture, it was not feasible to continue counting the number of oviposition holes to 2nd June 2023).

Commencing on 9th January 2023, the first F1 adult (i.e., offspring of the field-collected weevils) emerged at 68 days after first exposure to the fireweed plant. Thereafter, 148 F1 adults emerged, representing a viability rate of about 43 % of the original number of eggs laid (dated to 2nd March 2023). The average (± SD) number of days from oviposition to emergence of F1 adults was 77 (± 7). Commencing on 20th January 2023, the F1 adults were observed to have begun ovipositing on fireweed plants. Altogether, between 20th January and 30th March 2023, the cohort of F1 adults laid 821 eggs, with F2 adults now beginning to emerge.

In summary, these data indicate that the weevil colony has become well-established within the Australian quarantine facility, with a sustained pipeline of oviposition leading to emergence of healthy adults across multiple generations.

Table 2. Details of consignments of field-collected adult *G. tricostalis* weevils sent from the University of KwaZulu-Natal, South Africa to CSIRO, Canberra, Australia

Consignment number	Import permit (DoA)	Testing permit (DoE)	Milestone reporting year	Consignment date (pathway)	Collection sites	GPS		No. weevils sent	Arrival date	No. days to delivery	No. weevils alive on arrival	Colony status in Australian quarantine facility
BAA385917	0004821336	PWS2020-AU-002416	1	24th Feb 2021 (courier)	Ashburton Albert Falls Mpophomeni	29.6951°S 29.4638°S 29.5469°S	30.4955°E 30.3804°E 30.1822°E	5 5 10	2nd Mar 2021	6	10	Colony survived for approx. 150 days upon arrival, with high rate of fecundity for a few reproductively mature female weevils.
AEKTGC6YF	0004821336	PWS2020-AU-002416	1	26th Mar 2021 (courier)	Albert Falls	29.4638°S	30.3804°E	20	9th Apr 2021	14	0	Entire colony dead upon arrival, likely to due to extended time in transit.
AELHHYH79	0004821336	PWS2020-AU-002416	2	24th May 2021 (courier)	Albert Falls UKZN Gardens	29.4638°S 29.6251°S	30.3804°E 30.4040°E	5 15	3rd Jun 2021	10	11	Field-collected weevils began dying within a day of arrival, with the entire colony collapsing up to 50 days upon arrival of the consignment, with moderate levels of reproductive output.
BAA625798	0004821336	PWS2021-AU-001240	2	19th Nov 2021 (courier)	Albert Falls Ashburton	29.4638°S 29.6951°S	30.3804°E 30.4955°E	5 15	1st Dec 2022	12	2	Both adult weevils that survived the transit period were in poor condition and died within two days of arrival.
Q20276995	0004821336	PWS2021-AU-001240	2	12th Mar 2022 (hand delivered)	UKZN Gardens Ukulinga Ifafa Beach Ashburton	29.6251°S 29.6581°S 30.4633°S 29.6951°S	30.4040°E 30.4073°E 30.6522°E 30.4955°E	11 7 1 1	14th Mar 2022	2	20	Most adult weevils died within a few days upon arrival of the consignment, with all dead within two weeks of arrival with no evidence of reproductive output.
NAA22059912	0004821336, 0006636839	PWS2022-AU-001459	**3**	n/a (hand delivered)	Ukulinga Ashburton Albert Falls UKZN Mpophmemi	29.6581°S 29.6951°S 29.4638°S 29.6951°S 29.5486°S	30.4073°E 30.4955°E 30.3804°E 30.4955°E 30.1818°E	7 6 1 4 2	2nd Nov 2022 (hand delivered)	Overnight flight	20	Colony thriving, with 25 % of field-collected weevils still alive at 160 days after receipt. As of 2nd May 2023, a total of 148 F1 adults have been reared, with emergence of F2 adults (number not provided).
							Total No. weevils	**120**			63	

Figure 5. Stages in rearing *Gasteroclisus tricostalis* in quarantine within the CSIRO Black Mountain laboratories, Canberra: (a-b) receipt of first consignment in March 2021, (c) adult weevils copulating, (d) egg deposition hole plugged with adult frass in fireweed stem and (e) developing larvae in dissected fireweed stem.

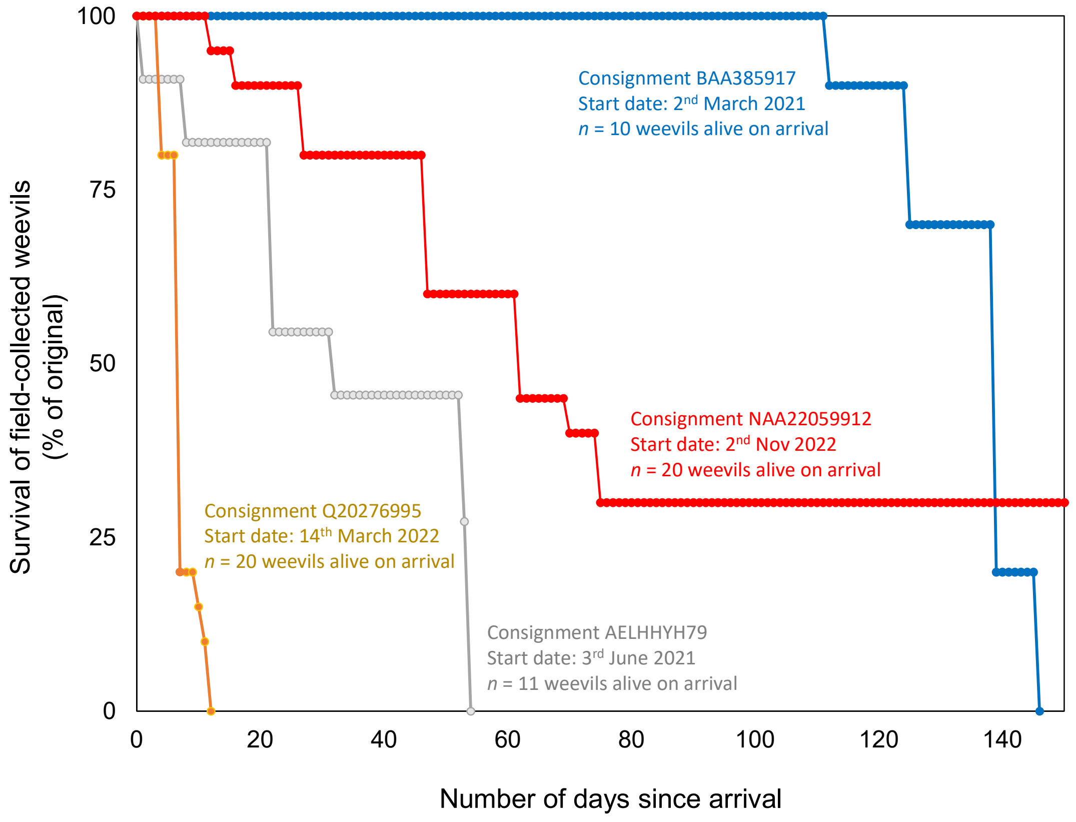


Figure 6. Survival rate for field-collected weevils shipped to Australian quarantine. Number of days were standardised to "0 day" from arrival of each consignment.

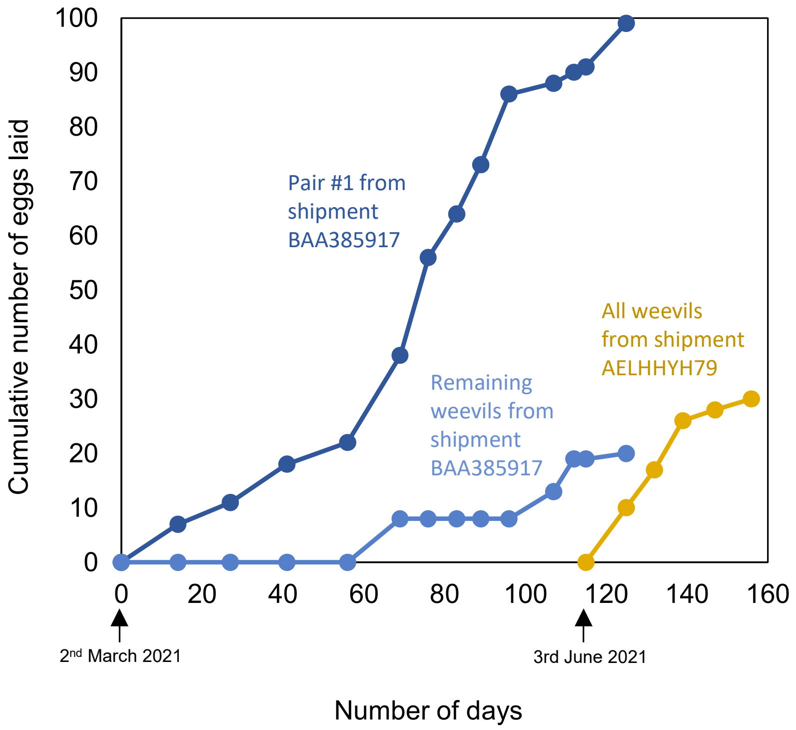


Figure 7. Cumulative number of eggs laid by field-collected weevils in Australia quarantine facilities. Note that cultures from shipments BAA385917 and AELHHYH79 were initiated on two different dates, three months apart. Also note that the dark blue curve represents cumulation of eggs laid by a single highly fecund female, which died after ~120 days. Note that the "0 day" is benchmarked on 2nd March 2021.

Figure 8. (a) Development of ‘galls’ at fireweed stem junctions, (b) in which late instar larvae were observed to pupate after tunnelling down the fireweed stem (from tip to root).

Table 3. Development durations for key larval and pupal stages for 25 larvae tracked daily for up to 140 days under quarantine conditions in Australia.

Individual larva	Trial ID	Parent ID	Date of plant exposure to adult weevils	Temperate (˚ C)	No. days to gall formation	No. days to pre-pupa development	No. days to pupa development	No. days to adult emergence
1	Cage 1 BAA385917	Pair #1 BAA385917	2/03/2021	17			132	133
2	Cage 2 BAA385917	Pair #1 BAA385917	16/03/2021	17			93	119
3	Cage 3 BAA385917	Pair #1 BAA385917	29/03/2021	17		119		
4	Cage 6 BAA385917	Combined cage BAA385917	10/05/2021	17			77	
5	Cage 8 BAA385917	Pair #1 BAA385917	24/05/2021	17		63		
6	Cage 8 BAA385917	Pair #1 BAA385917	24/05/2021	17		63		105*
7	Cage 9 BAA385917	Pair #1 BAA385917	30/05/2021	17		64		
				Average		**77.25**	**100.67**	**119.00**
				SD		**27.84**	**28.29**	**14.00**
8	Cage 13 BAA385917	Pair #1 BAA385917	25/06/2021	22		45	63	79*
9	Cage 13 BAA385917	Pair #1 BAA385917	25/06/2021	22		45		63
10	Cage 3 AELHHYH79	AELHHYH79 mixed	25/06/2021	22		45		74
11	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			88
12	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22		88		
13	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			92
14	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			92
15	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			92
16	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			102
17	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22	59			102
18	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22				102
19	Cage 4 AELHHYH79	AELHHYH79 mixed	5/07/2021	22				102
20	Cage 5 AELHHYH79 (gall#1)	AELHHYH79 mixed	12/07/2021	22	52		100	
21	Cage 5 AELHHYH79 (larva #2)	AELHHYH79 mixed	12/07/2021	22	52		101	
22	Cage 5 AELHHYH79 (adult #1 formed in stem)	AELHHYH79 mixed	12/07/2021	22	52			101
23	Cage 5 AELHHYH79 (adult #2 formed in stem)	AELHHYH79 mixed	12/07/2021	22				101
24	Cage 5 AELHHYH79 (adult #3 formed in stem)	AELHHYH79 mixed	12/07/2021	22				101
25	Cage 6 AELHHYH79	AELHHYH79 mixed	19/07/2021	22				104
				Average	**56.67**	**55.75**	**88.00**	**93.00**
				SD	**3.50**	**21.50**	**21.66**	**12.31**

* Not alive when emerged

3 Development of a draft host test list of Australian native and other commercially important plant species for host testing

3.1 Revision of phylogenetic associations between fireweed and native Australian *Senecio* species, with a focus on varieties within the native Australian *Senecio pinnatifolius* complex

Context

NB – section 3.1 has been published in the following international peer-reviewed journal, from which the subsequent text has been reproduced with modification: Schmidt-Lebuhn, A.N., Egli, D. and Gooden, B., 2022. Invasive *Senecio madagascariensis* Poir. and the *Senecio pinnatifolius* A. Rich. complex (Senecioneae): Evolutionary relationships and their implications for biological control research. *Capitulum*, 2(1), pp.20-37 (presented in full in **Appendix 2**).

Senecio pinnatifolius

Senecio pinnatifolius, formerly treated as *S. lautus* (Belcher 1994), has traditionally been considered a potential close relative of fireweed, *S. madagascariensis* (Prentis et al. 2007; Wijayabandara et al. 2022). Recent phylogenetic studies have demonstrated the two species to be very distantly related, showing fireweed as part of an African clade of *Senecio* and *S. pinnatifolius* as part of Australasian sequence clades (Liew et al. 2018; Schmidt-Lebuhn et al. 2020). A complicating factor is, however, that *S. pinnatifolius* is a morphologically and ecologically diverse species of currently eight recognised varieties (Thompson 2005a). Their delineation and the circumscription of the species complex as a whole remain controversial (Radford et al. 2004; Thompson 2005a) and have never been comprehensively studied using genetic data. This means that if the complex is in reality not a natural group but includes disparate lineages, the phylogenetic position of single samples may be misleading when preparing a host test list for fireweed biocontrol research in Australia.

Senecio pinnatifolius var. *alpinus* is an alpine taxon; var. *capillifolius* occurs on islands in the Bass Strait and has unusually finely divided leaves; var. *lanceolatus* occurs along the coasts of southeastern South Australia, Victoria, and northern Tasmania; var. *latilobus* occurs along the coast of southwestern Western Australia; var. *leucocarpus* is known from a single locality in Western Australia; var. *maritimus* occurs along the southern coasts of Australia and Tasmania; the most widespread and common var. *pinnatifolius* shares much of its distribution in eastern Australia with fireweed; and var. *serratus* is found in south-eastern Queensland and north-eastern New South Wales.

Our aim was to DNA-sequence the eight varieties of *S. pinnatifolius* and incorporate those new sequences into a revised phylogenetic analysis of the *Senecio* genus, to enable the refinement of species selection in the host test list developed during the first year milestone reporting period.

Sequencing methods

We generated Sanger sequence data for 38 leaf samples representing some *Senecio* taxa for which no data were previously available, species whose phylogenetic placement we wanted to confirm independently, and most taxonomic varieties of *Senecio pinnatifolius*. The targeted sequence regions and primers used were the same as in our previous study (Schmidt-Lebuhn *et al.* 2020); i.e., the nuclear ETS and ITS as well as chloroplast psbA-trnH and trnL regions, so that the new sequence data could be added to the existing phylogeny. For the purposes of the present report, we focus on ribosomal data (nrETS, nrITS), because they provide stronger phylogenetic resolution and confidence than the chloroplast regions.

DNA was extracted from herbarium specimens at Australian National Herbarium and John Bews Herbarium at the University of KwaZulu-Natal. Laboratory work and sequencing was outsourced to the Australian Genome Research Facility. Contigs were produced using Geneious (www.geneious.com), alignments were produced using MAFFT (Katoh and Standley 2013), and phylogenetic analysis was conducted with iqtree (Nguyen *et al.* 2015).

Results and implications for species selection in the host test list

The phylogenetic placement of re-sequenced species in their respective clades was confirmed (e.g., *Senecio inaequidens*, *S. harveianus*), and we were able to place additional internationally invasive species, such as *S. skirrhodon*, in the context of their evolutionary relationships as part of the same African clade as fireweed (**Figure 9**).

The native Australian varieties of *Senecio pinnatifolius* did not form a single clade, suggesting that the species complex as currently circumscribed is not a natural group. *Senecio pinnatifolius* var. *pinnatifolius*, var. *alpinus*, var. *lanceolatus*, var. *latilobus*, and var. *maritimus* formed part of Australasian clade 3, thus confirming their membership of the 'lautusoid group' as described by Liew *et al.* (2018). Inside this clade they were, however, split into two groups: var. *pinnatifolius* and var. *alpinus* were placed as sister to *S. spathulatus*, whereas var. *lanceolatus*, var. *latilobus*, and var. *maritimus* were grouped with *S. hamersleyensis*, *S. lacustrinus*, *S. spanomerus*, and *S. warrenensis*. Our priority for host-specificity testing will thus be on var. *pinnatifolius* and var. *alpinus*.

Surprisingly, *Senecio pinnatifolius* var. *capillifolius* and var. *serratus* were placed in an otherwise African clade along with e.g., *S. crassiflorus* and *S. panduriformis*. However, consultation with South African taxonomists specialising in Asteraceae did not produce a match with any known African *Senecio* species (Marinda Koekemoer, pers. comm.). Although these results suggest that *Senecio pinnatifolius* as currently circumscribed is unlikely to be a natural unit, let alone a single species, further research is needed to suggest a more appropriate taxonomy.

Furthermore, of all species presumed native to Australia, *Senecio condylus* was placed closest to fireweed in previous phylogenetic analyses by Schmidt-Lebuhn *et al.* (2020). It was described as new to science only as recently as 2005 and considered endemic to southwestern Western Australia (Thompson 2005b). However, its placement as part of an otherwise entirely South African clade (Schmidt-Lebuhn *et al.* 2020) raised the question of whether it represents a native Australian species that arose from a recent natural trans-continental dispersal event or rather a novel weed arising from human-assisted introduction from South Africa, like fireweed itself. Sandra Williamson of the Western Australian DBCA has since confirmed that *S. condylus* is a junior synonym of a South African species that has been introduced to Australia and is preparing a manuscript of her findings (Williamson & Balkwill, unpublished data). This species will therefore not be considered as a top priority native Australian species for use in host specificity experimentation with the candidate biocontrol agent *G. tricostalis*.

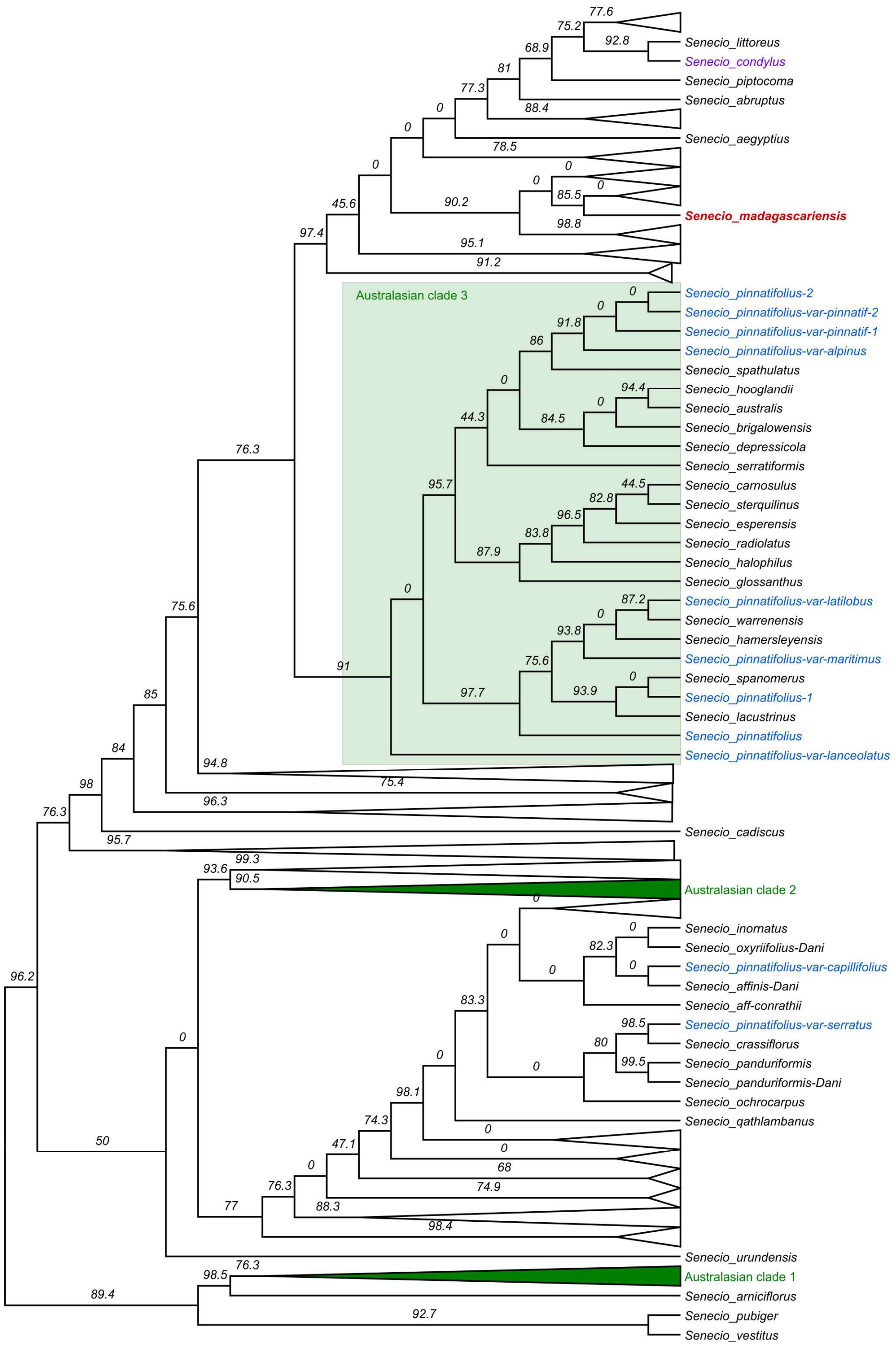


Figure 9. Ribosomal phylogeny of Senecio showing relationships of fireweed (red, bold font) to Australasian clades (green), S. condylus (purple), and different varieties of S. pinnatifolius in its current circumscription (blue). Numbers above branches indicate clade support values. Triangles indicate clades of multiple species that have been collapsed to allow the display of the relationships relevant to this report in a single figure.

3.2 Phylogenetic analysis suggests uncertain genetic associations between fireweed in South Africa, New Zealand, and Australia

Context

The aim of this study was to confirm the assumed origin (KwaZulu-Natal, South Africa) of fireweed in Australia and to verify the presence for the first time of fireweed in New Zealand. This analysis of genetic data, which has been subsequently published (see reference below), was undertaken as part of the broader phylogenetic analysis of the associations between fireweed and native Australia *Senecio* species underpinning the development of a host test list (section 3.1). The results of this analysis will help to refine the source location of the weevil *Gasteroclisus tricostalis* for ongoing host-specificity testing.

NB – section 3.2 has been published in the following international peer-reviewed journal, from which the subsequent text has been reproduced with modification: Schmidt-Lebuhn, A.N., Egli, D., Grealy, A., Nicholls, J.A., Zwick, A., Dymock, J.J. and Gooden, B., 2022. Genetic data confirm the presence of *Senecio madagascariensis* in New Zealand. *New Zealand Journal of Botany*, pp.1-13 (presented in full in **Appendix 3**).

Introduction

The *Senecio inaequidens* – *S. madagascariensis* complex (Asteraceae: Senecioneae, subsequently "fireweed complex") is a group of six southern African species generally assumed to be closely related and often described as being difficult to distinguish morphologically and potentially taxonomically unresolved (Marohasy 1993; Radford et al. 2000). They are *S. inaequidens* DC., *S. madagascariensis* Poir., *S. skirrhodon* DC., *S. harveianus* MacOwan, *S. burchellii* DC., and *S. pellucidus* DC. Representatives of this complex are invasive weeds in various parts of the world, including Mexico (Rzedowski et al. 2003), South America (López et al. 2008), Japan (Tsutsumi 2011), Australia (McFadyen and Morin 2012; Wijayabandara et al. 2022), the Hawaiian Islands (Le Roux et al. 2006), and New Zealand (Webb et al. 1988) (**Figure 10**).

A monograph of the Asteraceae of KwaZulu-Natal, South Africa, (Hilliard 1977) has since its publication served as the authoritative taxonomic treatment of the fireweed complex, despite its restricted geographic scope, which also means that it does not provide descriptions of *S. burchelli* and *S. pellucidus* and only a rudimentary description of *S. harveianus*. According to Hilliard's key and descriptions, *S. skirrhodon* is a coastal plant with fleshy leaves, *S. madagascariensis* an annual branching mostly in the upper part of the stem, and *S. inaequidens* and *S. harveianus* are perennials branching vigorously from the base. The latter two can be distinguished by their calycular bracts (few and short versus several rows and long). *Senecio burchellii* and *S. pellucidus* are easily distinguished from the other four species by only possessing c. 12 involucral bracts and 5–8 ray florets versus c. 20 and c. 13, respectively.

However, Hilliard's work has some limitations, in particular if it is used without access to reference specimens identified by that author. Not all potentially useful characters from the descriptions are used in the key. For example, the couplet differentiating *S. skirrhodon* from other species requires the user to know if the species has fleshy leaves, which is difficult to determine from dried herbarium specimens, or if it would be growing on a beach in its natural habitat, which is not necessarily useful in invasive populations outside of its native range. In addition, some characters such as the number and dimensions of calycular bracts are not quantified.

Partly because of these limitations, partly because of the morphological plasticity of the species, especially with regard to growth form and leaf shape, taxonomists and weed researchers in the introduced range have struggled with the identification of invasive fireweed populations and relating them to South African taxa to study their invasion histories and source candidate biological control agents. European fireweed populations introduced with South African wool were variously named *S. harveianus*, *S. reclinatus* L.f., and *S. vimineus* DC., but are now generally named *S. inaequidens* (Jovet et al. 1975; Tutin et al. 1976). Argentinean fireweed was first described as a new species, then identified as *S. burchelli* (Cabrera and Ré 1965), and is now named *S. madagascariensis*. In Mexico, it was misidentified twice, until comparison with European *S. inaequidens* revealed the two as likely being the same species (Rzedowski et al. 2003). Australian fireweed was long considered part of the native *S. pinnatifolius* A.Rich. (formerly *S. lautus* Willd.) species complex. It was identified as *S. madagascariensis* in 1980 (Michael 1981) and only widely accepted as introduced after genetic confirmation (Scott et al. 1998).

Recently, uncertainty has arisen about the identity of fireweed populations in New Zealand, which have historically all been assigned to *S. skirrhodon* (Webb et al. 1988). Increased concern about the weediness of *Senecio* species in pastures in the Northland region of New Zealand prompted one of the authors (J.J.D.) to lodge specimens in the Allan Herbarium CHR in May 2017. They were tentatively identified as *S. madagascariensis*, with the New Zealand online flora listing the species as present (Schönberger et al. 2020). A recent review of the distribution of *S. madagascariensis* considered it to be absent from New Zealand but noted that most of it would be suitable habitat (Wijayabandara et al. 2022).

Previous attempts at clarifying the identity of invasive fireweed populations or pinpointing their provenances have used isozymes (Radford et al. 2000), chromosome counts and genome sizes (Lafuma et al. 2003), microsatellites (Dormontt et al. 2014), Amplified Fragment Length Polymorphism data, and ribosomal Internal Transcribed Spacer sequences (Le Roux et al. 2006; Lachmuth et al. 2010), all of which have limited power of resolution and frequently produce ambiguous results such as large phylogenetic polytomies. Today, high-throughput sequencing and the use of methods such as sequence capture allow the use of marker systems that provide considerably more sequence data and therefore more resolution and higher clade support in phylogenetic analyses (Koenen et al. 2020; Schmidt-Lebuhn and Bovill 2021).

In this study, we used sequence capture to produce data for hundreds of low-copy nuclear genes to test the hypothesis that two fireweed species occur in New Zealand. In addition, we sampled South African, European, and Australian fireweed occurrences to provide a phylogenetic context for the New Zealand samples and develop a first framework dataset that can be built upon for future research on the taxonomic identity and provenance of other fireweed populations globally.

Materials and methods

Sampling for this study focused on populations identified as *Senecio madagascariensis*, *S. skirrhodon*, and *S. inaequidens* in South Africa, Australia, New Zealand, and Europe. South African specimens were collected through dedicated field work in the KwaZulu-Natal area (**Figure 10**), which was previously suggested as the likely area of origin of invasive *S. madagascariensis* in the

Hawaiian Islands and Australia (Scott et al. 1998; Le Roux et al. 2006), covering multiple locations and morphological forms; vouchers are deposited at the John Bews Herbarium, University of KwaZulu-Natal (NU). New Zealand specimens were collected in dedicated field work to represent the two forms of fireweed suggested to occur in the country from different parts of the North Island (**Figure 13**); vouchers are deposited at the Australian National Herbarium (CANB). These specimens are distinct from the three specimens previously deposited at CHR. Australian and European populations were sampled mostly from herbarium specimens at CANB, aiming for broad geographic coverage, including different European countries (**Figure 10**). One specimen was newly collected and is vouchered at the CSIRO European Laboratory.

To place the fireweed populations into a phylogenetic context, we sampled other representative species of *Senecio* from South Africa and added data of Asteraceae tribe Senecioneae available from the Plant and Fungal Tree of Life (PAFTOL) and Genomics for Australian Plants (GAP) consortia.

Genomic DNA was extracted from 5–15 mg silica dried leaf tissue or herbarium material using Invisorb Spin Plant Mini Kit (Stratec, Berlin, Germany) following the manufacturer's instructions. Libraries were built using the QIAseq FX DNA Library UDI-A Kit 96 (Qiagen, Clayton, Australia) that included a DNA digestion step to a fragment size of approximately 200 base pairs, and sequence capture was conducted on pools of sixteen libraries using the angiosperms353 (Johnson et al. 2019) MYbaits kit (Daicel Arbor Biosciences, Ann Arbor, MI, USA). Enriched libraries were sequenced on Illumina NovaSeq 6000 SP with v1.5 paired-end 2 × 150 cycle chemistry.

Reads were quality filtered and paired with TRIMMOMATIC (Bolger et al. 2014) and assembled using hybpiper-nf (Jackson et al. 2021), a Nextflow pipeline adapted from HybPiper (Johnson et al. 2016), against a target file designed for broad representation of Asteraceae by mining transcriptome data for angiosperms353 targets (McLay et al. 2021).

The results of HybPiper's paralog finder were analysed with the Monophyletic Outgroups (MO) algorithm as implemented in resolve_paralogs-nf (Jackson et al. 2021), a Nextflow pipeline for the four gene-tree based paralogy resolution algorithms collated by Yang and Smith (2014). We chose this algorithm because it returns at most one ortholog group for each locus, producing a more complete sample × gene matrix than alternative algorithms that return more ortholog groups with on average fewer sequences. After paralogy resolution, 326 ortholog groups were retained. Of these, eleven were discarded because they had data for less than ten terminals, leaving 315 that were analysed. Terminals were represented in 25–314 ortholog groups (mean 266), and ortholog groups had data for 10–209 terminals (mean 181).

Custom-written Python scripts were used to ensure that gene alignments were in frame and to concatenate them into a supermatrix. The concatenated dataset comprised 210 terminals and 165,504 characters, of which 63,814 were parsimony informative, 34,943 variable but uninformative, and 66,747 constant.

To explore the potential role of reticulate evolution in our data, we inferred locus heterozygosity and allele divergence for the samples of the fireweed complex using HybPhaser (Nauheimer et al. 2021).

A phylogeny of the concatenated supermatrix was inferred with IQTREE 2.2.0.5 (Minh et al. 2020), under automatic partition- and model-testing (Lanfear et al. 2017), with codon positions as the starting partitions. The favoured model maintained the partition scheme with GTR+F+I+G4 as the substitution model in all partitions. UltraFast Bootstraps (UFB) were used to estimate branch support (Minh et al. 2013). The phylogeny was outgroup-rooted on the genus Abrotanella, which is often placed as sister to the remainder of Senecioneae (Zhang et al. 2021).

Results

Deeper relationships in the phylogeny were largely consistent with topologies previously inferred from nuclear ribosomal data. The samples from the fireweed complex and South African *Senecio leptophyllus* DC. Formed a clade with high support (UFB 100).

Within the complex, three main clades were resolved (**Figure 11**): First, *S. inaequidens* from Europe, *S. leptophyllus*, and *S. harveianus*, with only weak support (UFB 61). Most internal relationships in this clade were also poorly supported, including the clade of *S. inaequidens*. Second, a very strongly supported clade (UFB 100) of South African *S. skirrhodon* and two New Zealand samples from the southeast of North Island. Third, a strongly supported (UFB 95), large clade of all South African and Australian specimens identified *a priori* as *S. madagascariensis*, several specimens of uncertain affiliation from South Africa, and nine New Zealand samples from Northland. Within *S. madagascariensis*, all Australian and New Zealand specimens (except two) formed a strongly supported (UFB 95) clade, and all South African specimens and two Australian specimens formed its sister clade, albeit with weak support (UFB 82).

To support our re-identification of specimens of doubtful affiliation, we compiled information from Hilliard's descriptions and key and quantified some additional characters by examining photos of type specimens and specimens identified by Hilliard. Morphological re-examination of the South African specimens of originally uncertain identification in the *S. madagascariensis* clade against Hilliard's specimens and descriptions made their placement in that clade plausible, as they showed typical branching patterns and leaf shapes. New Zealand specimens placed in *S. skirrhodon* showed leaf length to width ratios at the upper end of the range expected for this species and higher than other specimens of the species, presumably because specimens of uncertain affiliation were preferentially collected for sequencing in this study. However, they also had calyculus bracts that were larger and in particular wider (c. 0.8–1.5 mm) than those placed in *S. madagascariensis* (c. 0.5–0.8 mm) (**Figure 12**), in both cases matching the respective species' type material.

Field observations by one of the authors and collectors' notes on herbarium specimens indicate that the two species tend to occupy different habitats in New Zealand. *Senecio madagascariensis* was found in grazed pasture, whereas *S. skirrhodon* occurs in beach habitats such as dunes but also variously on roadside gravel and lava tracks, along railway lines and streams, and on wasteland and building sites.

Discussion

Our data add to growing confidence that sequence capture data from hundreds of genes can provide phylogenetic or population genetic resolution even at the level of species complexes (Nicholls et al. 2015; Slimp et al. 2020; Schmidt-Lebuhn 2022). As results are based on larger numbers of sequence characters and gene regions, they can provide more confidence in species delimitation and relationships than historically used marker systems such as nuclear ribosomal spacer regions (Radford et al. 2000; Le Roux et al. 2006).

By sampling four of the six species of the fireweed complex, we resolved three main lineages. Two of them are individual species (*S. madagascariensis, S. skirrhodon*), and the third comprises three species (*S. inaequidens, S. harveianus, S. leptophyllus*). Most critically, New Zealand samples could be placed with very high confidence in two of these clades, *Senecio madagascariensis* and *S. skirrhodon*. The confirmation of the identity of *S. madagascariensis* in northern regions of New Zealand supports recent field observations of the invasiveness of *Senecio* species in pasture habitats there. *Senecio skirrhodon* does not display this invasive behaviour in New Zealand.

Within *S. madagascariensis*, two subclades were observed, albeit with one of them poorly supported. All New Zealand samples of the species and most Australian samples formed one clade, and South African samples and two Australian samples formed the second. This suggests three possible conclusions. First, the species may have been introduced to Australia at least twice, as suggested previously (Dormontt et al. 2014). Second, it is possible that New Zealand populations are derived from Australia, given that they are nested inside the latter. however, with our current geographic coverage, this conclusion must remain tentative.

This is because of the third conclusion, i.e., that KwaZulu-Natal, to which our South African sampling was limited, may not represent the area of origin of most introduced Australasian populations. Historically, that area has been considered the most likely area of origin of introduced *S. madagascariensis* based on isozyme data and ribosomal sequence similarity (Scott et al. 1998; Radford et al. 2000; Le Roux et al. 2006). However, the sister group relationship between samples from KwaZulu-Natal and nearly all introduced samples does not support this assumption, because we would in that case expect the latter to be phylogenetically nested inside the former. Further southern African sampling is needed to test the second and third conclusions and hence resolve the origins of New Zealand populations.

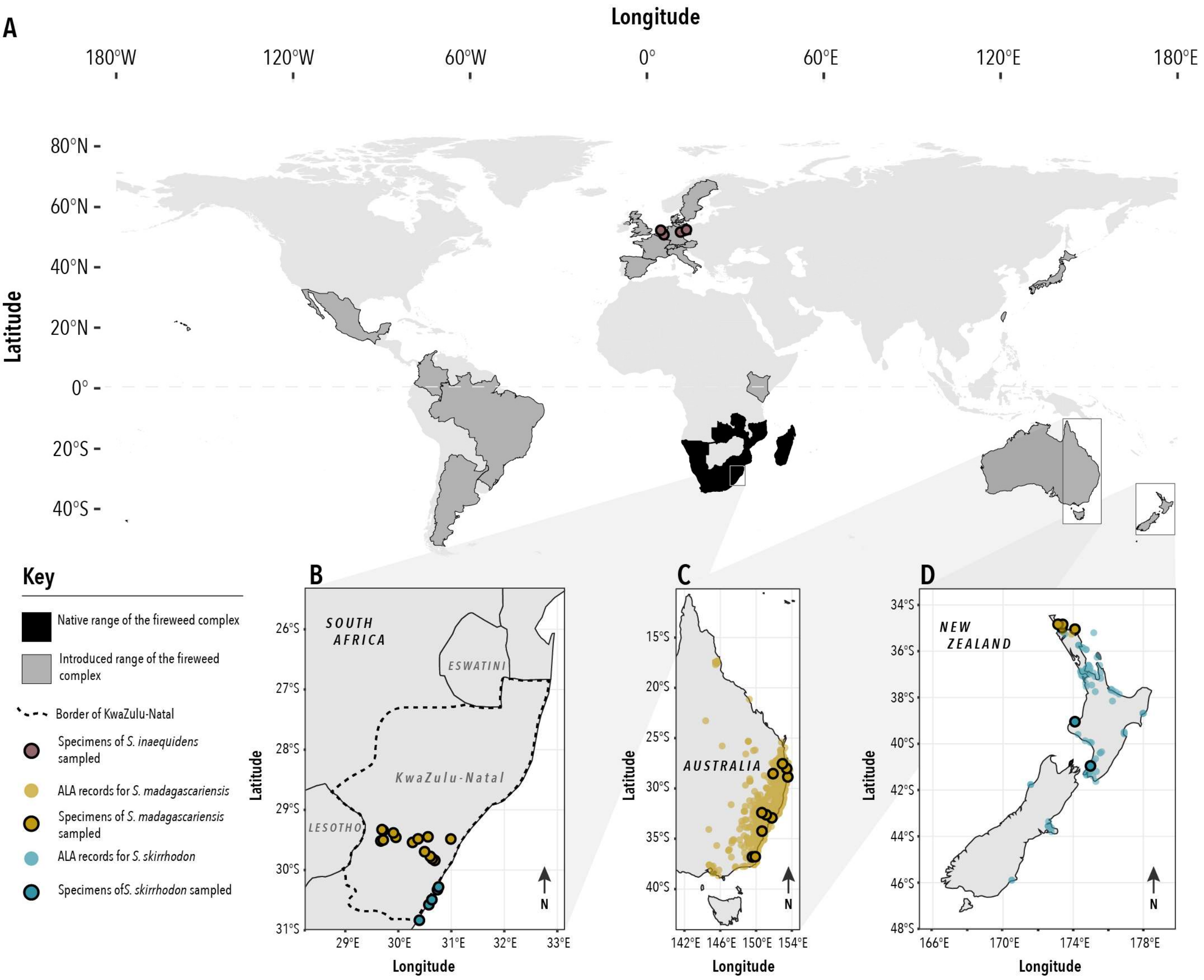


Figure 10. A. Countries representing the native (black) and approximate introduced (dark grey) range of the fireweed complex. B. Sampling localities in KwaZulu-Natal, South Africa. C. Introduced range and provenance of specimens sequenced in Australia. D. Introduced range and sampling localities in New Zealand. Senecio inaequidens is marked brown, S. skirrhodon blue, and S. madagascariensis yellow. Points with black margin indicate sequenced samples, those without margin geocoded specimens in the Australasian Virtual Herbarium (accessed September 2022).

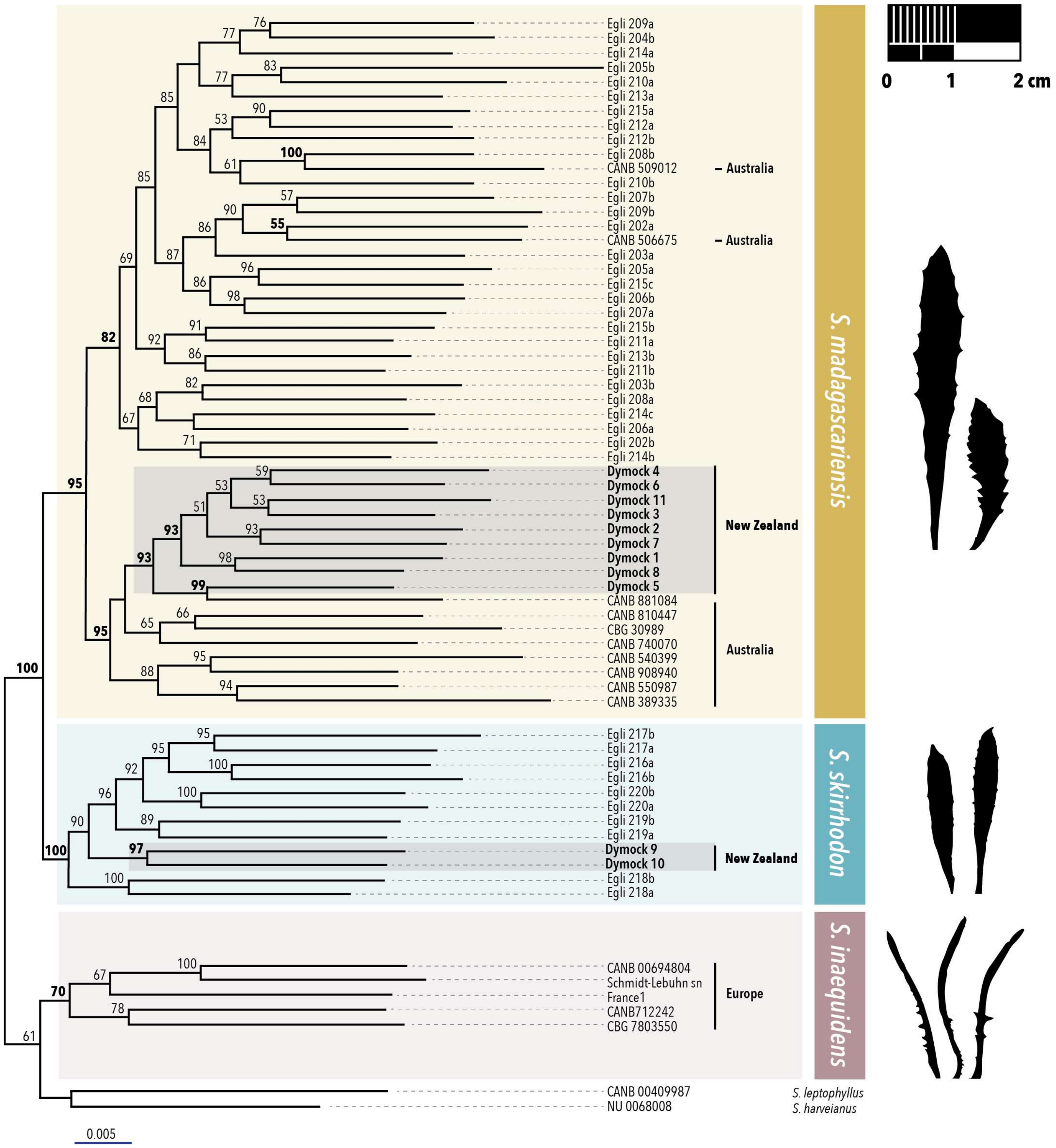


Figure 11. Part of the phylogenetic tree of Senecioneae showing the fireweed complex. Three main clades are highlighted with boxes: Senecio inaequidens (brown), S. skirrhodon (blue), and S. madagascariensis (yellow). Silhouettes show representative leaf shapes of type specimens. Branch labels are UltraFast Bootstrap values.

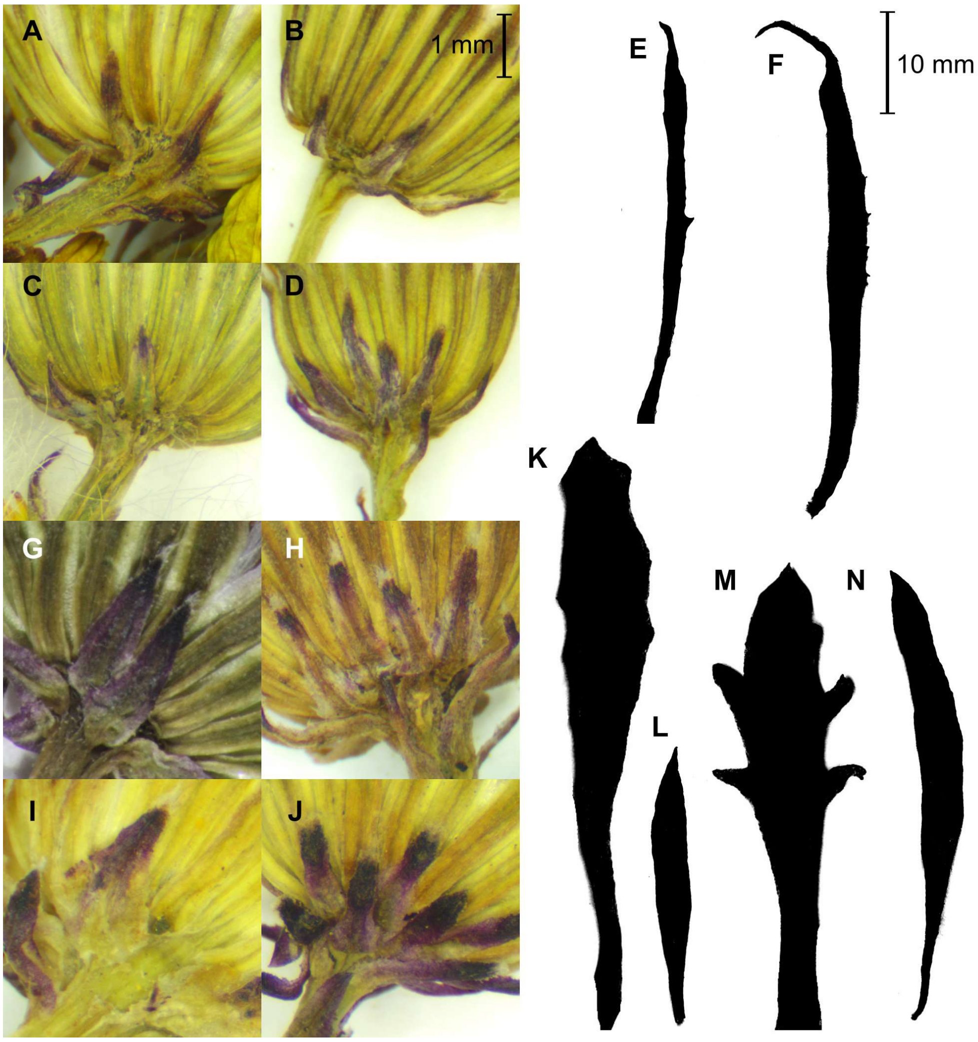


Figure 12. Comparison of calycular bracts and leaves of Senecio madagascariensis (A.-F.) and S. skirrhodon (G.-N.) in New Zealand. Calyculus images are all to same scale, as are leaf silhouettes. A. J.J. Dymock s.n. (CANB 953777), B. J.J. Dymock s.n. (CANB 953779), C. J.J. Dymock s.n. (CANB 9537780), D. J.J. Dymock s.n. (CANB 9537783), E. J.J. Dymock s.n. (CANB 953779), F. J.J. Dymock s.n. (CANB 953783), G. W. Symes s.n. (CHR 649039A), H. M. Gray 7108 (CANB 508525), I. J.J. Dymock s.n. (CANB 953781), J. J.J. Dymock s.n. (CANB 953782), K. W. Symes s.n. (CHR 645039B), L. J.J. Dymock s.n. (CANB 953782), M. W. Symes s.n. (CHR 645039A), N. J.J. Dymock s.n. (CANB 953781).

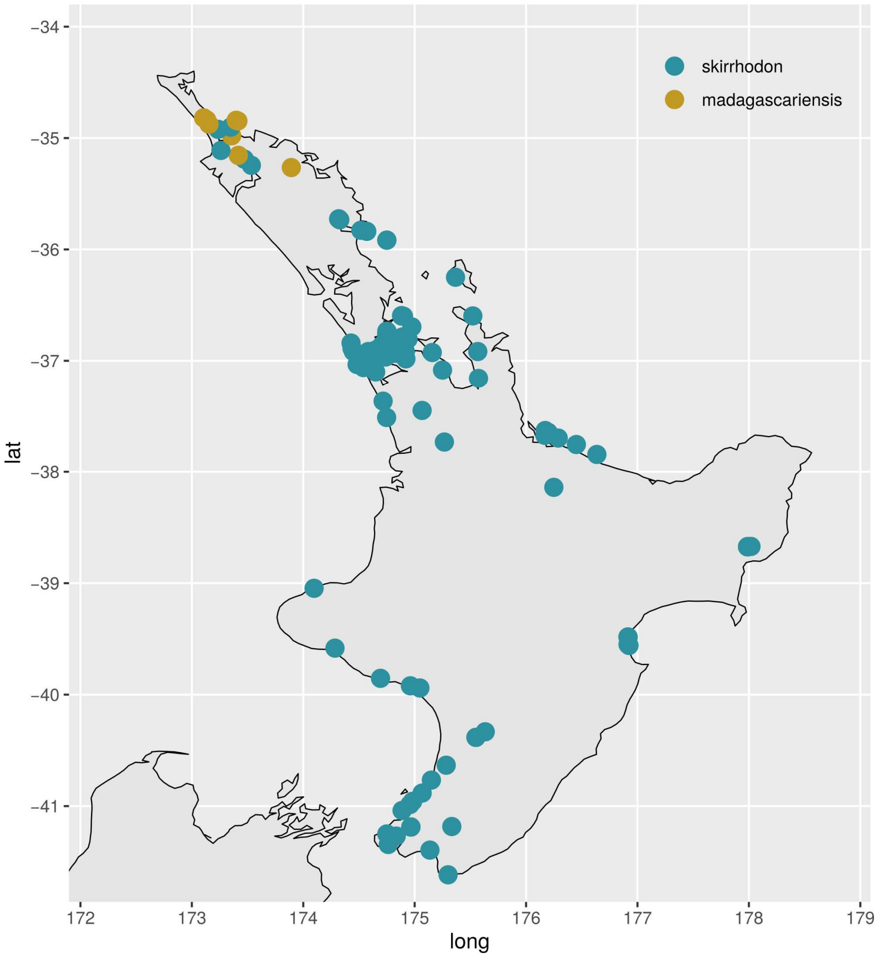


Figure 13. Detailed map of the North Island of New Zealand showing geocoded specimens of *Senecio madagascariensis* (yellow) and *S. skirrhodon* (blue) from the Australasian Virtual Herbarium (accessed October 2022). One specimen with coordinates in the ocean has been removed.

3.3 Expanded genetic data help refine the phylogenetic associations amongst fireweed populations at a global scale

Introduction

In May-June 2023, we undertook a second phylogenetic analysis, which expanded upon the dataset presented under 3.2 (above) for New Zealand and Australian samples of fireweed (*S. madagascariensis*) and the closely related taxon, *S. skirrhodon*. Given that our first analysis of Australian and New Zealand fireweed provenances showed southern African and invasive populations as sister clades, we concluded that our sampling in southern Africa, which was biased towards KwaZulu-Natal, was insufficient to identify the area of origin.

The aim of the expanded analysis was therefore to resolve the likely source location in the native range of invasive fireweed populations in Australia and New Zealand by expanding the range over which the species were sampled in South Africa (the native range) and other introduced populations in Japan, Hawaii, and South America. The results of the second analysis will help to refine the source location of the weevil *Gasteroclisus tricostalis* for ongoing host-specificity testing, especially if the genetic data indicate that KwaZulu-Natal is not likely to be the source location of fireweed invasion into Australia, as suggested previously (Scott et al. 1998; Le Roux et al. 2006).

Methods

We expanded our sampling for this second analysis by requesting additional material covering wider geographic provenances from the herbarium of the University of KwaZulu-Natal (NU) and the herbarium of Sydney (NSW). In addition, we expanded our sampling to include specimens from Argentina (also provided by NU), Japan (herbarium TNS), the Hawaiian Islands (Bishop Museum, BISH), northern Queensland outlier populations (Queensland Herbarium, BRI), and the incursion into Melbourne (Australian National Herbarium, CANB). We intended to also include Uruguayan specimens, but they arrived too late to be included in sequencing.

In total, we intended to sample another 34 specimens of *Senecio madagascariensis*, six of *S. inaequidens*, and three of *S. harveianus*. However, re-identification of specimens found in unexpected positions after the phylogenetic analysis revealed that several of them had been misidentified. Our expansion therefore added one specimen that we would now identify as *Senecio burchellii*, four of *S. harveianus*, five of *S. inaequidens*, three of *S. skirrhodon*, and only 30 of *S. madagascariensis*. Most specimens supposedly of *Senecio madagascariensis* from the Cape region of South Africa were misidentified, leaving only one to represent that area for the species. Locations of all new specimens collected from southern Africa and Australia are provided in **Figure 14**.

Molecular methodology was as described by Schmidt-Lebuhn et al. (2022) under section 3.2 above, except that lab work was conducted at the Australian Genome Research Facility using full-size library prep reactions. Bioinformatic and phylogenetic methodology was as described by Schmidt-Lebuhn et al. (2022) under section 3.2 above, except for the following differences in dataset specifics:

- After paralogy resolution, 320 ortholog groups were retained. Of these, eight were discarded because they had data for less than ten terminals, leaving 312 that were analysed. Terminals were represented in 21–311 ortholog groups (mean 254.5), and ortholog groups had data for 10–366 terminals (mean 302.7). None of the specimens of the fireweed complex had data for fewer than 160 genes, so that it is unlikely that unexpected phylogenetic placement is due to poor data quality (i.e., not excluding the possibility of lab error or cross-contamination).
- The expanded concatenated dataset in this case comprised 371 terminals and 164,397 characters, of which 71,758 were parsimony informative, 34,717 variable but uninformative, and 57,921 constant.

Results

Many deeper relationships inside the fireweed clade did not receive strong support, but there was strong support for three species (*Senecio harveianus*, *S. madagascariensis*, and *S. skirrhodon*) and two infraspecific lineages in *S. madagascariensis* (**Figure 15**). The clade of *S. harveianus*, which was previously only sampled with a single specimen (see section 3.2), now comprises four specimens, one of which was previously identified as *S. inaequidens*. It was maximally supported with 100% ultra-fast bootstrap (UFB).

The clade of *Senecio skirrhodon* was accidentally expanded by three South African specimens from Eastern Cape that had been misidentified as *S. madagascariensis*. Closer examination of their label data revealed that all three were collected in beach or river mouth habitats typical of *S. skirrhodon*, and one specified “leaves subfleshy”, a useful character that is, unfortunately, lost on dried specimens. The species was strongly supported as a clade (UFB 96%) and was again placed as sister to *S. madagascariensis*, albeit with weak support (UFB 57%).

Senecio madagascariensis as currently understood was strongly supported as a clade (UFB 97%). It was divided into two large clades. The first comprised all southern African specimens from KwaZulu-Natal province, Mpumalanga province, and Eswatini, as well as two Argentinean specimens and one Australian specimen from the Hunter Valley (UFB 99%). The second clade comprised all other invasive populations of fireweed from the Hawaiian Islands, Japan, Australia, and New Zealand, and a single South African specimen collected from the Eastern Cape. Except for all New Zealand specimens (except one) forming a clade (UFB 99%), there was no clear geographic structure for the invasive populations, with provenances from different countries mixed up together and the single South African sample deeply nested within the clade. Outside of these three species, phylogenetic placements were less well supported.

Discussion

Despite our efforts to expand the geographic coverage across southern Africa to include Western and Eastern Cape provinces, Mpumalanga province, and Madagascar, the provenance of most invasive populations of *Senecio madagascariensis* remains uncertain. This is mostly due to many relevant specimens having turned out to be misidentified, and in part due to the uncertain placement of our only sample from Madagascar. Nevertheless, our expanded dataset provided new insights beyond the previously published study as presented in section 3.2. above (Schmidt-Lebuhn et al., 2022).

The strongly supported separation of all invasive fireweed (*S. madagascariensis*) populations (except the Argentinean ones) from all samples from KwaZulu-Natal and neighbouring Mpumalanga makes it increasingly unlikely that the invasive populations originated in those regions of South Africa. The single correctly identified specimen of *S. madagascariensis* from Eastern Cape was placed in the invasive clade with high support, suggesting the possibility that this province is its area of origin in Australia, New Zealand, Hawaii and Japan. However, before that conclusion can be drawn with confidence, additional correctly identified samples of *S. madagascariensis* from both Eastern and Western Cape and Madagascar should be added to the dataset. We have already reached out to two herbaria to obtain additional samples.

Two of the three Argentinean samples of *S. madagascariensis* were placed in the predominantly South African clade. This suggests that South American populations may be distinct from those that have invaded Japan, the Hawaiian Islands, Australia, and New Zealand. In a future expansion of the dataset, we will add Uruguayan specimens to increase confidence in this conclusion. Conversely, the addition of Japanese and Hawaiian samples has provided strong evidence that they and the Australian and New Zealand populations of *S. madagascariensis* constitute a single genetic lineage. The presence of a single fireweed specimen from the Hunter Valley (NSW, Australia) in the South African clade (alongside a specimen collected from Ashburton in KwaZulu-

Natal province), suggests there may be multiple introduction events and source populations for fireweed in Australia.

Regarding the natural range of *S. skirrhodon*, Hilliard (1970) wrote, “along the seashore from Madagascar and Mozambique as far south as Humansdorp district in the southern Cape if *S. litorosus* Fourcade … can be equated with it.” Regarding its circumscription, Hilliard speculated that it was “possibly no more than a maritime form of *S. madagascariensis*”. The placement of succulent-leafed specimens from beaches in the Eastern Cape province in the *S. skirrhodon* clade with high confidence provides evidence both that the species indeed occurs as far south as suggested by Hilliard and that it is distinct from *S. madagascariensis*. The strong support for the *S. madagascariensis* and *S. skirrhodon* clades as being distinct despite both including specimens from Eastern Cape and KwaZulu-Natal makes it very unlikely that *S. skirrhodon* is merely an ecotype of *S. madagascariensis*.

Specimen data available on the aggregator GBIF (gbif.org) indicate that southern African *S. madagascariensis* occurs from around the Cape of Good Hope in the west to Mozambique and Madagascar in the east. However, visual examination of specimens and photos of supposed *S. madagascariensis* from Western Cape province suggests that most, if not all, of those specimens may be misidentified, and it is possible that the species does not occur that far to the west (Ben Gooden, pers. obs.). Furthermore, given the large proportion of Eastern Cape specimens that were found to be misidentified, we conclude that many specimens of *S. madagascariensis* from the Eastern Cape region may be misidentified, and current distribution maps are unreliable.

Whereas most samples of *S. harveianus* form a maximally supported clade (with only a single sample placed in an isolated position with weak support), the delineation of *S. inaequidens*, including from *S. harveianus*, remains less well resolved. Both species are very similar, sharing a perennial habit and narrow, linear to lobed leaves. They differ in that *S. harveianus* has several rows of calycular bracts, the longest of which reach half the length of the involucre, whereas *S. inaequidens* has only few, short calycular bracts. However, we observed that European populations of *S. inaequidens* showed rather long calycular bracts compared to Hilliard’s (1970) descriptions (Schmidt-Lebuhn et al., 2022). As observed by Lafuma et al. (2003), at the time on the basis of genome size variation, the delimitation of the species may require taxonomic attention.

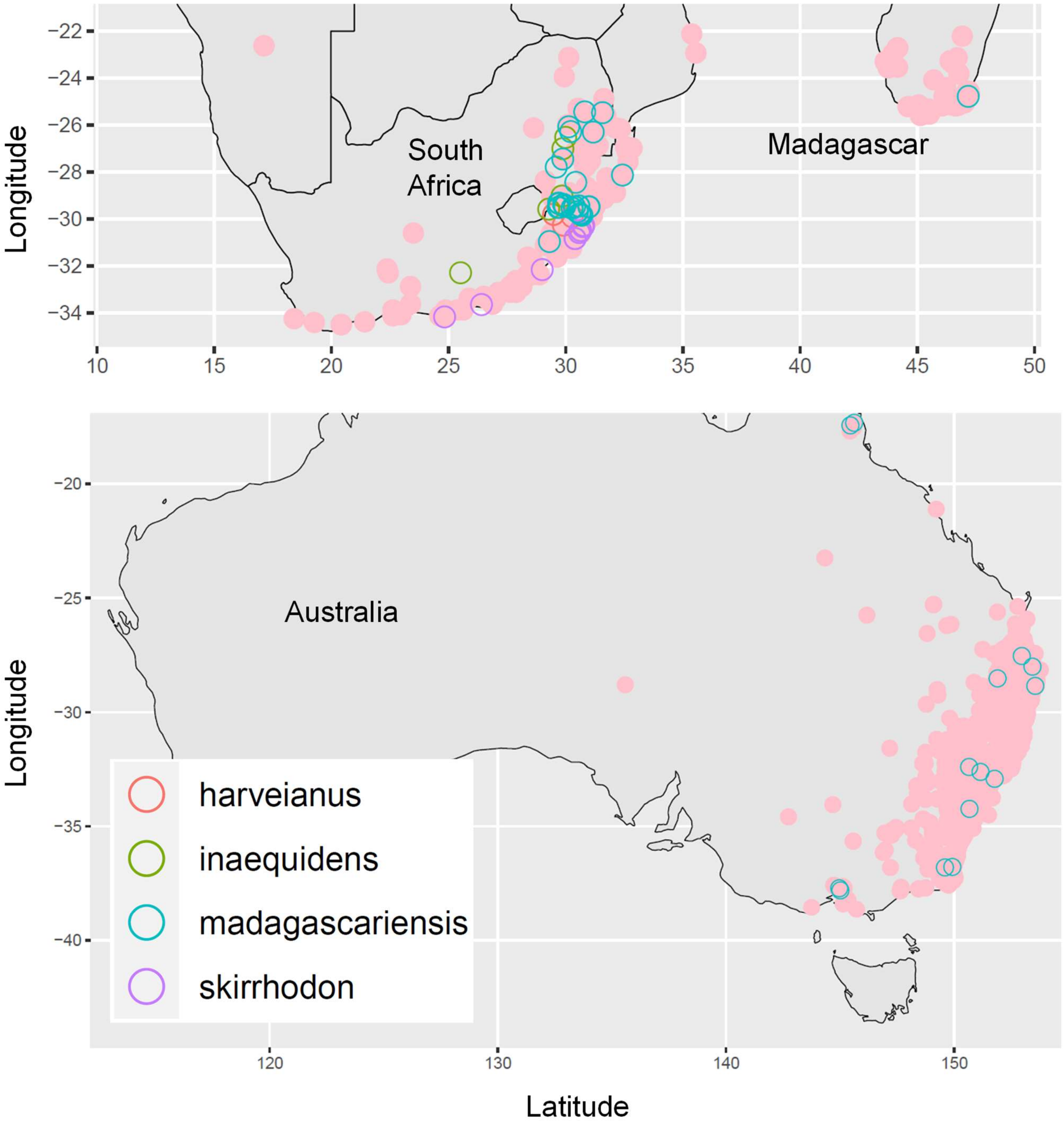


Figure 14. Maps of southern Africa and Australia showing geocoded specimens (open circles) of the extra *Senecio* specimens (for four species) included in the expanded phylogenetic analysis. Closed pink circles represent recorded sightings of fireweed (*S. madagascariensis*) obtained from the Global Biodiversity Information Facility.

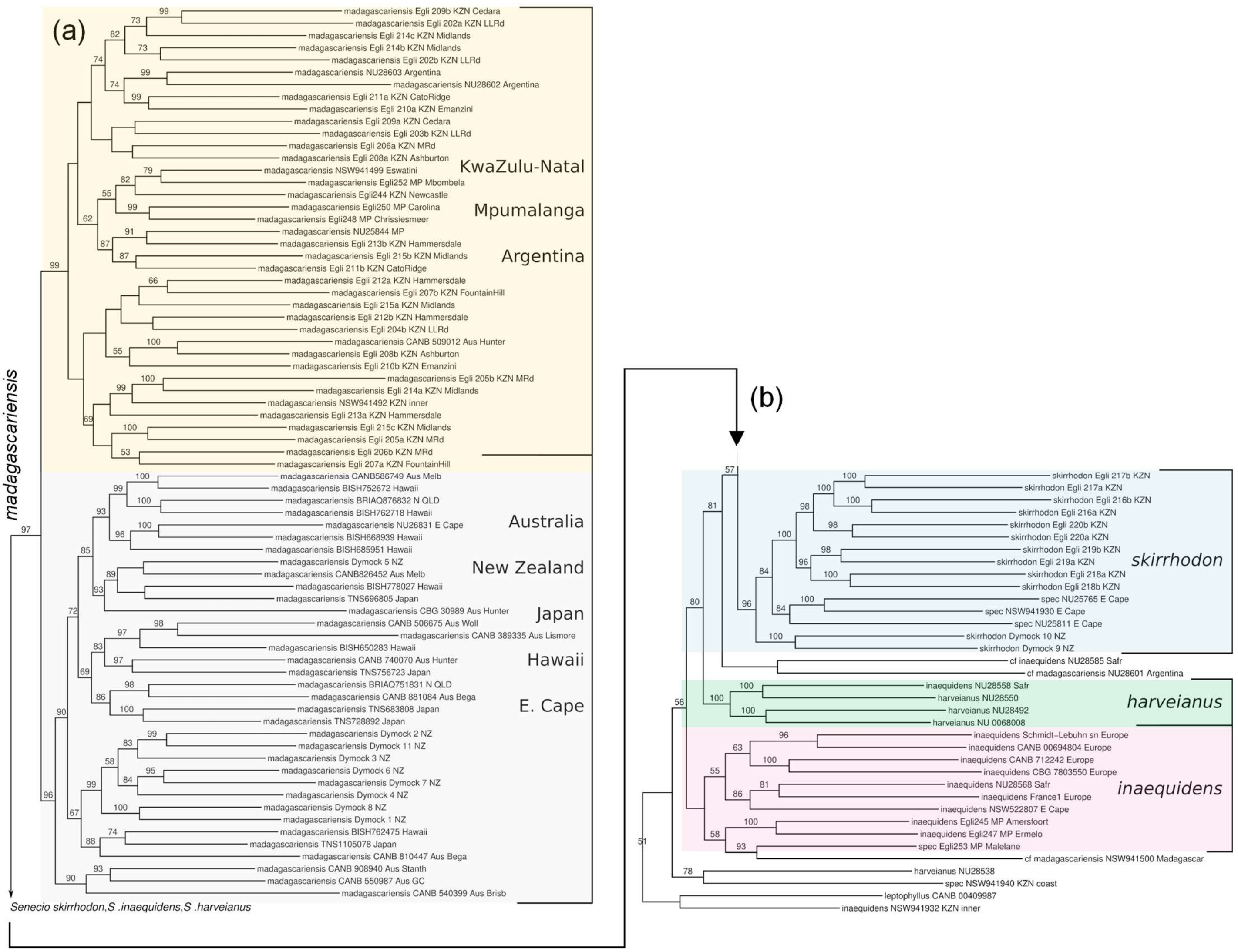


Figure 15. Part of the phylogenetic tree of Senecioneae showing the fireweed complex. Five main clades are highlighted with boxes: (a) *Senecio madagascariensis* of mostly southern African and Argentinean origin in yellow, and all other introduced populations plus a single sample from Eastern Cape in grey; (b) *S. skirrhodon* in blue, *S. harveianus* in green and *S. inaequidens* in pink. Branch labels are UltraFast Bootstrap values. Each specimen is accompanied by the relevant herbarium accession code and location.

3.4 Publication of draft host test list

In January 2021, a draft host test list was prepared and submitted to The Australian Department of Agriculture, Water and the Environment for public comment. The following text is derived from the information package that was put together to explain the methods of host test list development and rationale for selection of non-target host-test species as part of the public consultation process. Citation: Gooden B, Egli D, Kaye L, Schmidt-Lebuhn AN (2021) Proposed plant host test list for assessing risk of biological control agents for fireweed, *Senecio madagascariensis* Poir. CSIRO, Australia. The published version is presented in full in **Appendix 4**.

Target weed – fireweed (*Senecio madagascariensis*)

Fireweed is a significant weed of coastal and temperate rangeland pastures, roadsides, and other disturbed habitats across south-eastern Australia. It was first recorded in Australia in the lower

Hunter Valley region of NSW in the 1910s, and is thought to have been introduced via shipping (Parsons and Cuthbertson 2001; Sindel 2009; Sindel 1986). Fireweed has since spread northwards into south-eastern Queensland and southwards along the New South Wales coastline, including the Northern and Southern Tablelands of NSW (Parsons and Cuthbertson, 2001; Sindel, 2009). More recently, it has spread further along the far south coast of NSW, where it is now considered one of the worst pasture weeds in the Bega Valley region (Sindel, 2009; Sindel et al., 2011; Sindel et al., 2008). Climate suitability modelling and evidence of continued spread suggests that fireweed has not yet reached its maximum potential geographic extent in Australia, despite significant investment in chemical and manual (i.e. hand-pulling) control by regional councils and local land holders (McFadyen and Morin 2012; Sindel and Michael 1992; Sindel et al. 2008).

Candidate biocontrol agent – stem-boring weevil *Gasteroclisus tricostalis*

Gasteroclisus tricostalis ex. *Senecio madagascariensis* (family Curculionidae, order Coleoptera) is a stem-boring weevil associated with fireweed in its native range of KwaZulu-Natal, South Africa (Egli and Olckers 2020, Egli et al. 2020). Both adults and larvae can be found in the field throughout the year but peak in abundance during summer and are almost absent in areas where host fireweed populations die back over winter (Singh 2019). Multiple larvae have been found feeding within the stems of a single fireweed plant, with a mean of 3 and a maximum of 21 larvae per plant (Egli and Olckers 2020). Both the larvae and adults of *G. tricostalis* feed on the vegetative structures of fireweed. After hatching, the larvae bore down into the stem towards the root crown, feeding internally on structural tissue. When larvae are larger and nearer to the base of the plant, they can completely hollow-out the stem’s central pith, which may lead to stem collapse. Adult weevils feed externally on leaf tissue of the same host plant, although adult feeding damage is minor relative to larval damage.

Gasteroclisus tricostalis was first identified as a candidate biocontrol agent following native range surveys of fireweed in the KwaZulu-Natal region of South Africa in 1989 and 1991 (Marohasy 1989; Marohasy 1991). *Gasteroclisus tricostalis* has been identified as one of the most promising candidate biocontrol agents on fireweed (Egli and Olckers 2015; Egli and Olckers 2020). During native range surveys between 2011 and 2017, researchers determined that *G. tricostalis* is strongly host specific to the fireweed species complex and causes extensive damage to host plant stems (Egli and Olckers 2015; Egli and Olckers 2020). Furthermore, curculionids (i.e. weevils) are historically some of the most successful biocontrol agents (Winston et al. 2014), and stem-boring curculionids have featured in the biocontrol of invasive Asteraceae species (Egli and Olckers 2017; Winston et al. 2014), setting a promising precedent for *G. tricostalis* on fireweed in Australia.

Development of the proposed host test list

The following proposed plant list has been developed based on the most recent revised classification of Compositae (Asteraceae) by Susanna et al. (2020) and phylogenetic analysis of Senecioneae, the largest tribe within Asteraceae of which fireweed is a member, by Schmidt-Lebuhn et al. (2020). The Asteraceae family in Australia comprises approximately 1,000 native species from about 200 genera (Brown, 2020), as well as hundreds of introduced species, many of which have become invasive. Fireweed is a member of the tribe Senecioneae. Australia has approximately 96 native and 22 non-native species of Senecioneae, with most belonging to the genus *Senecio* (Thompson and Orchard 2015).

Here, we present a proposed host test list containing up to 40 non-target plant species for inclusion in host specificity experiments, ranked in order of phylogenetic (i.e. evolutionary) distance to fireweed (**Table 4**). The selection of these test plants was based on the centrifugal phylogenetic method, which posits that plant species relatively closely related to the target weed are more likely to be attacked by the candidate biocontrol agent than more distantly related species (Briese 2003; Gilbert et al. 2012; Wapshere, 1974). Species were furthermore selected at each phylogenetic distance level based on biogeographical overlap and functional similarity with fireweed (e.g. growth form, reproductive life history, habitat preferences), with a focus on endemic native Australian species (Briese 2005).

The recent study by Schmidt-Lebuhn et al. (2020) placed fireweed within a phylogenetic framework with Australian native *Senecio* species. Their study identified the following three main clades (i.e. groupings of multiple species with recent shared common ancestry) containing native Australian *Senecio* species ranked in order of evolutionary distance to fireweed: (i) “Australasian Clade 3”, identified as the group of native *Senecio* species most closely related to fireweed (including *S. pinnatifolius*, *S. spanomerus*, *S. spathulatus*); (ii) “Australasian Clade 1” (including *S. linearifolius, S. bathurstianus, S. hispidulus*); (iii) “Australasian Clade 2” (including *S. gunnii, S. prenanthoides, S. quadridentatus*). Testing will also be undertaken for representative species from up to six tribes most closely related to Senecioneae; up to three subfamilies within Asteraceae; and up to three families most closely related to Asteraceae (**Table 4**).

Proposed plant host test list

Table 4. List of proposed plant species to be involved in testing the proposed biological control agent, the stem-boring weevil *Gasteroclisus tricostalis*, for the target weed *Senecio madagascariensis*. Species distributions are provided in Figures 16, 17 and 18.

Family	Subfamily	Tribe	Subtribe	Species	Phylogenetic distance from target weed [a]	Clade [b]	Status
ASTERACEAE	**ASTEROIDEAE**	**SENECIONEAE**	**SENECIONINAE**	*Senecio madagascariensis*	0	Unassigned	Target weed
				Senecio condylus [c]	4	Unassigned	Non-native, Naturalised
				Senecio glossanthus	7	Australasian Clade 3	Native
				Senecio spanomerus	7	Australasian Clade 3	Native
				Senecio brigalowensis	7	Australasian Clade 3	Native
				Senecio spathulatus	7	Australasian Clade 3	Native
				Senecio pinnatifolius	7	Australasian Clade 3	Native
				Senecio biserratus	13	Australasian Clade 1	Native
				Senecio distalilobatus	13	Australasian Clade 1	Native
				Senecio bathurstianus	13	Australasian Clade 1	Native
				Senecio glomeratus	13	Australasian Clade 1	Native
				Senecio macrocarpus	13	Australasian Clade 1	Native
				Senecio linearifolius	13	Australasian Clade 1	Native
				Senecio hispidulus	13	Australasian Clade 1	Native
				Senecio diaschides	13	Australasian Clade 1	Native
				Senecio minimus	13	Australasian Clade 1	Native
				Senecio bipinnatisectus	13	Australasian Clade 1	Native
				Senecio macranthus	16	Australasian Clade 2	Native
				Senecio gunnii	16	Australasian Clade 2	Native
				Senecio prenanthoides	16	Australasian Clade 2	Native
				Senecio tenuiflorus	16	Australasian Clade 2	Native
				Senecio quadridentatus	16	Australasian Clade 2	Native
				Senecio phelleus	16	Australasian Clade 2	Native
				Arrhenechthites mixta	19	Unassigned	Native

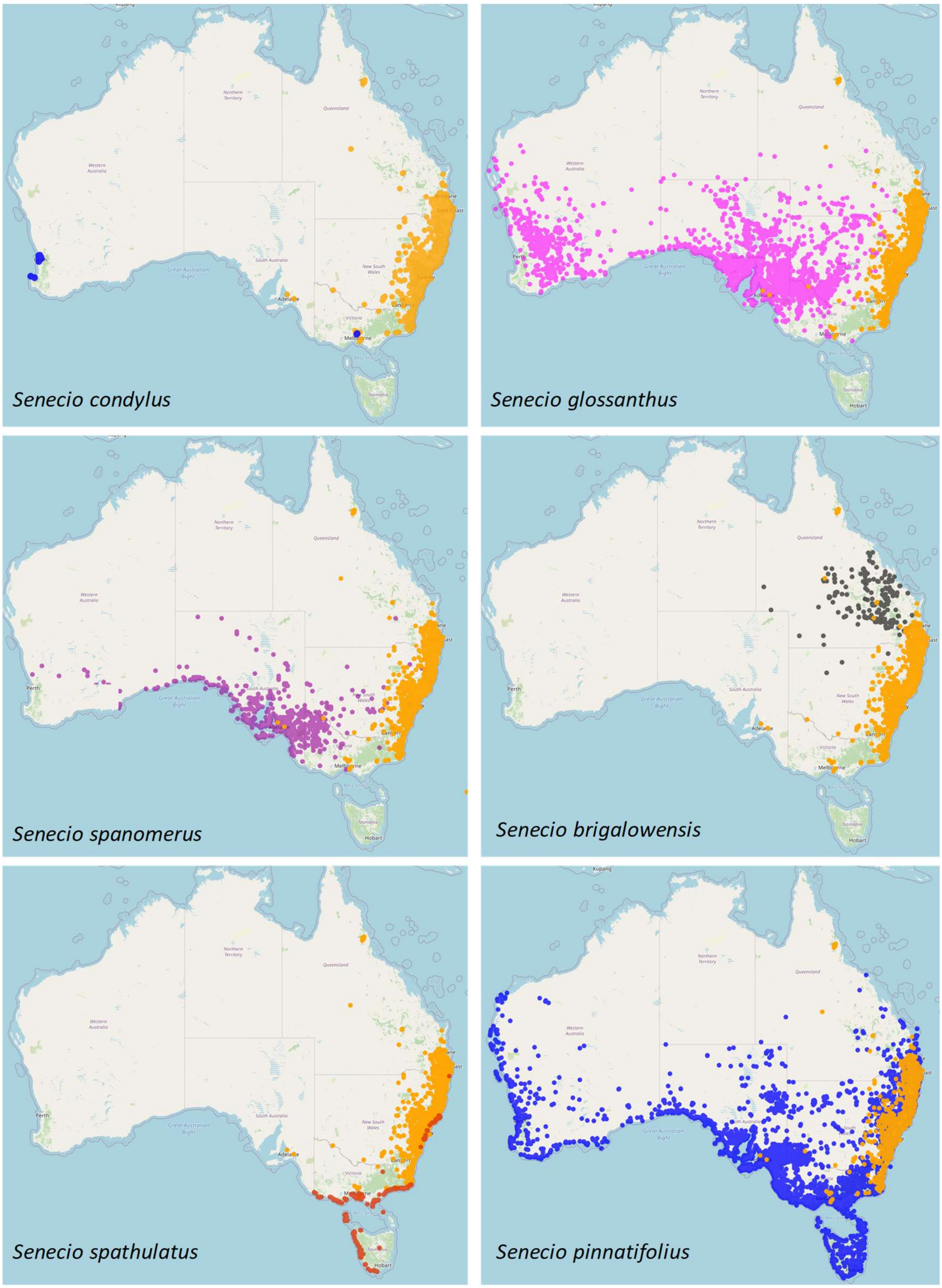
				Senecio vagus	19	Unassigned	Native
				Senecio velleioides	23	Unassigned	Native
			TUSSILAGININAE	*Bedfordia sp.*	27	n/a	Native
		ASTEREAE	**HINTERHUBERINAE**	*Olearia sp.*	32	n/a	Native
			BRACHYSCOMINAE	*Brachyscome sp.*	32	n/a	Native
		GNAPHALIEAE	**GNAPHALIINAE**	*Xerochrysum sp.*	32	n/a	Native
		ANTHEMIDEAE	**COTULINAE**	*Cotula sp.*	30	n/a	Native
		ATHROISMEAE	**CENTIPEDINAE**	*Centipeda sp.*	35	n/a	Native
		HELIANTHEAE	**HELIANTHINAE**	*Helianthus (sunflower) cultivar*	35	n/a	Non-native, Crop, Ornamental
		CALENDULEAE	**N/A**	*Calendula sp.*	33	n/a	Non-native, Ornamental
	CICHORIOIDEAE	**CICHORIEAE**	**LACTUCINAE**	*Lactuca sp.*	37	n/a	Crop
	VERNONIOIDEAE	**ARCTOTIDEAE**	**ARCTOTIDINAE**	*Cymbonotus lawsonianus*	38	n/a	Native
		VERNONIEAE	**ERLANGEINAE**	*Cyanthillium cinereum*	38	n/a	Native
	CARDUOIDEAE	**CARDUEAE**	**CARDUINAE**	*Cynara* (artichoke) cultivar	40	n/a	Crop
GOODENIACEAE	N/A	N/A	N/A	*Goodenia* sp.	n/a	n/a	Native
CAMPANULACEAE	N/A	N/A	N/A	*Wahlenbergia* sp.	n/a	n/a	Native
CAMPANULACEAE	LOBELIOIDEAE	N/A	N/A	*Lobelia* sp.	n/a	n/a	Native

a Phylogenetic distance from target weed: Values within the tribe Senecioneae (i.e. 4, *S. condylus* to 27, *Bedfordia* species) represent degrees of phylogenetic separation on the tree of evolutionary relationships published by Schmidt-Lebuhn et al. 2020. They count the number of intermediate evolutionary lineages separating the test species from the target species, starting with zero for the immediate sister lineage of the target and then counting by one for each ancestral lineage divergence. Values for other tribes within Asteraceae (i.e. 32, Astereae to 40, Carduoideae) were inferred from a recent classification of the plant family by Susanna et al. 2020 by similarly counting ancestral divergence events from the deepest split inside Senecioneae.

b Clade: Native Australian *Senecio* species were assigned to Australasian Clades 1-3 following Schmidt-Lebuhn et al. 2020, based on ribosomal sequences.

c *Senecio condylus*: The species was until recently considered native by Western Australian Flora (https://florabase.dpaw.wa.gov.au/browse/profile/25878), following a revised taxonomy of the *Senecio pinnatifolius*/*S. lautus* complex by Thompson (2005b). A recent review of the distribution, habitat preferences and occurrence records for *S. condylus* from the Swan River coastal plain around Perth by Keighery and Keighery (2017) suggested that it is a non-native, naturalised *Senecio* species native to either South Africa or Europe that proliferates in disturbed habitats in urban areas. This is consistent with molecular phylogenetic analyses undertaken by Schmidt-Lebuhn et al. 2020, which places *S. condylus* in a clade alongside *S. maritimus* (http://pza.sanbi.org/senecio-maritimus) and *S. littoreus* (http://pza.sanbi.org/senecio-littoreus) that are native to coastal habitats in Western Cape, South Africa. More recently, *S. condylus* has been found to be a species introduced from South Africa (Williamson, manuscript in prep.), and is thus considered non-native for this host test list.

Geographical distribution of test species



Senecio condylus

Senecio glossanthus

Senecio spanomerus

Senecio brigalowensis

Senecio spathulatus

Senecio pinnatifolius

Figure 16. Geographical distribution of the target weed (fireweed, *Senecio madagascariensis*; orange points) and six test plant species within the tribe Senecioneae, positioned at phylogenetic distance levels 4 (*S. condylus*) and 7 (all remaining species, members of Australasian Clade 3). Occurrence data from ALA, 5/1/2021

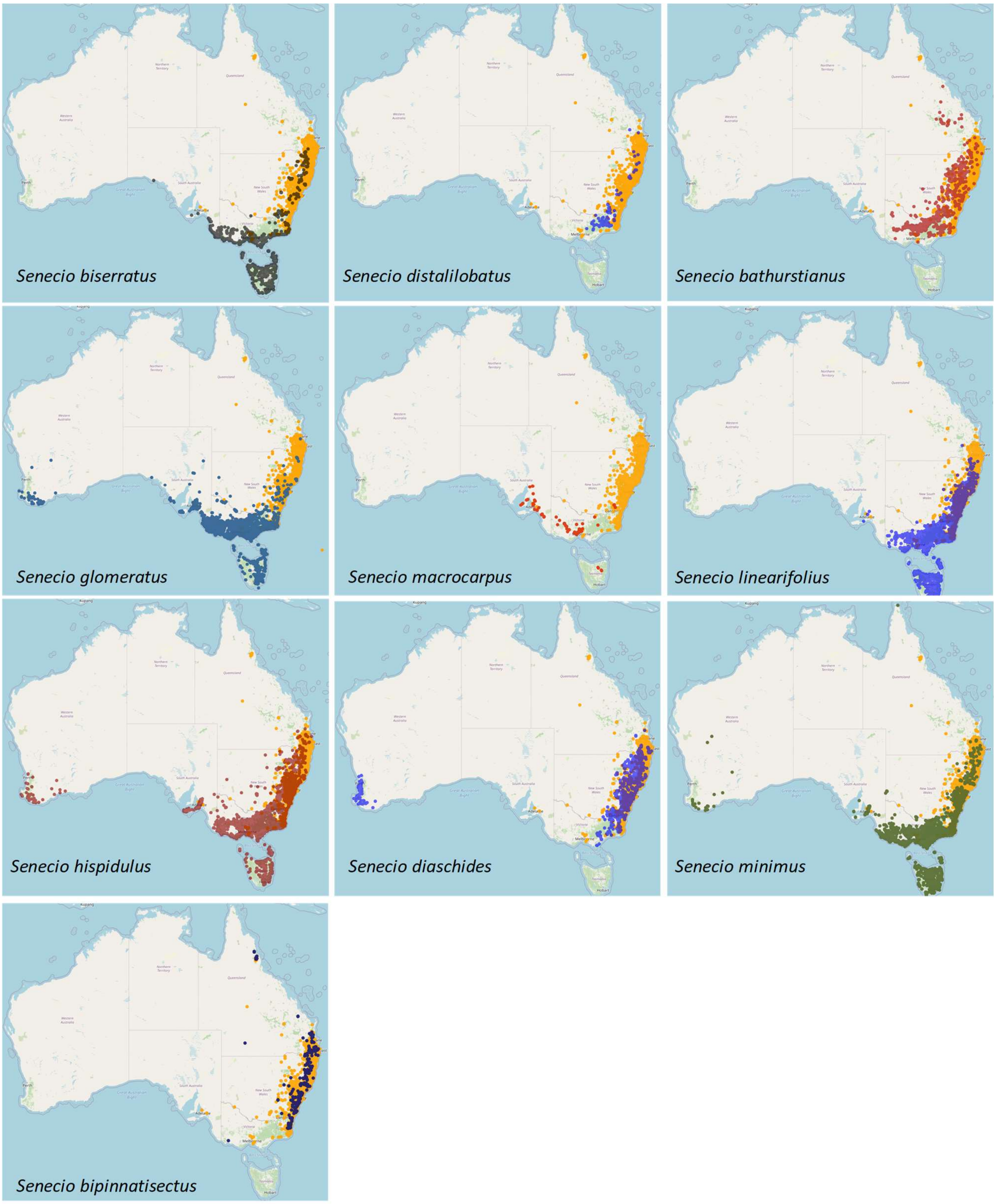


Figure 17. Geographical distribution of the target weed (fireweed, *Senecio madagascariensis*; orange points) and 10 test plant species within the Australasian Clade 1, all positioned at phylogenetic distance level 13. Occurrence data from ALA, 5/1/2021

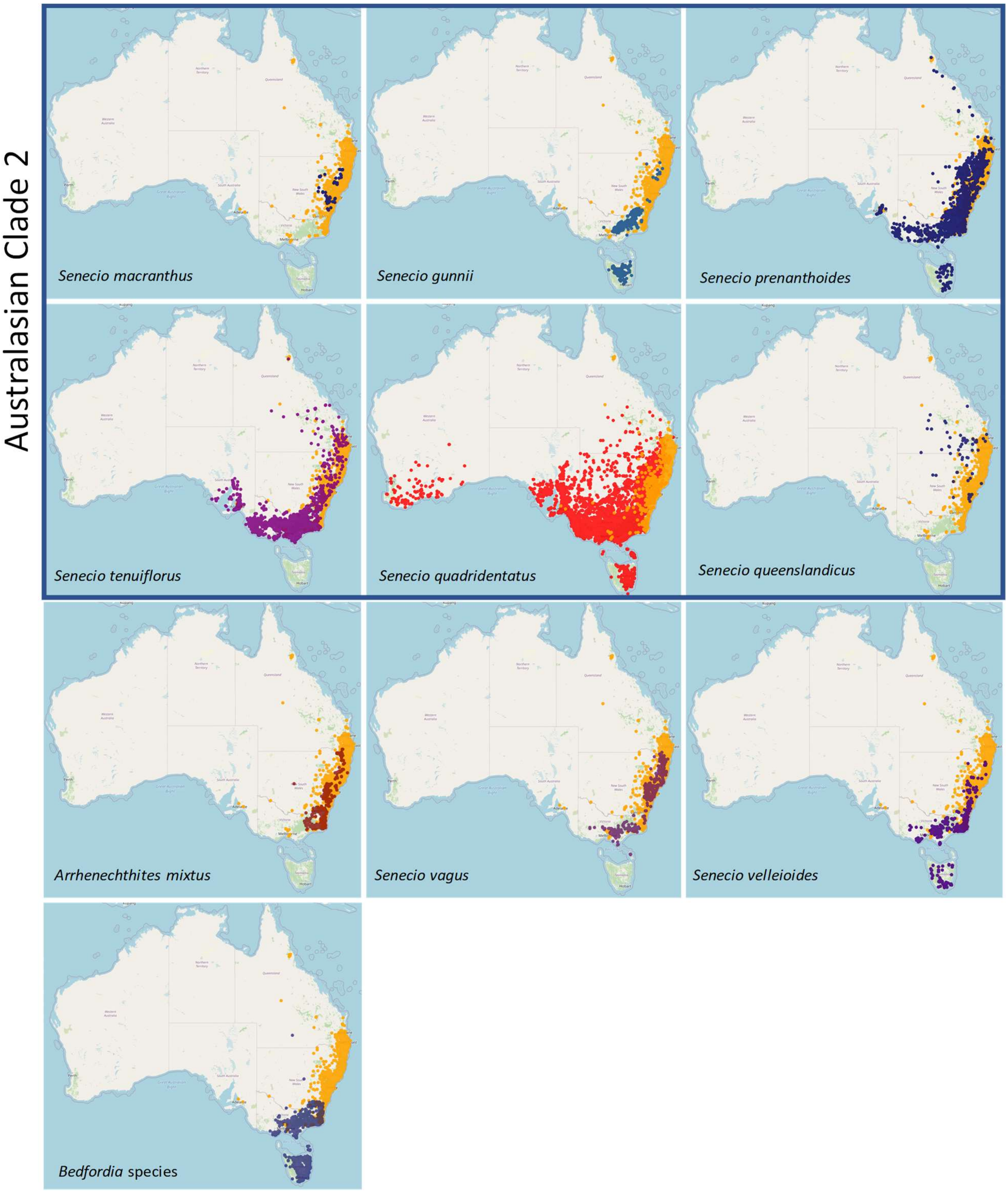


Figure 18. Geographical distribution of the target weed (fireweed, *Senecio madagascariensis*; orange points) and six test plant species within the tribe Senecioneae, positioned at phylogenetic distance level 16 (Australasian Clade 2) and four other plants species (phylogenetic distance levels 19-27). Occurrence data from ALA, 5/1/2021

4 Design and implementation of host specificity experiments to evaluate the risks of non-target damage of *Gasteroclisus tricostalis* to Australian native (and other economically and horticulturally important) plant species

4.1 Sourcing seed accessions for native *Senecio* species used in host-specificity experiments

Altogether, we successfully sourced seeds for most of the priority *Senecio* species (and other genera) included on the original host test list; specifically, a total of 80 accessions representing 33 unique plant taxa (**Table 5**). We also sourced seed for 10 populations of fireweed, representing the full range of its distribution in south-eastern Australia: the Bega Valley, Northern Rivers, and southeast Queensland. Several taxa (e.g., *S. condylus*, *S. glossanthus*, etc.) could not be sourced and were exchanged for other species (where possible at an equivalent phylogenetic distance level).

4.2 Supply of native Australian *Senecio* seeds to UKZN for host-specificity experimentation

In July 2022, two accessions of each of nine native Australian plant species were sent to UKZN from Australia (**Table 5**). These arrived safely and were planted in August 2022. Of these, eight accessions of six species germinated, though viability was generally very low (between 1 and 5 seedlings each). Most plants subsequently died but five individuals of two species (*S. hispidulus* and *S. diaschides*) survived and were successfully deployed in host-specificity trials (see section 4.3). Seeds from a further 27 accessions of 14 species were sent to UKZN from CSIRO in October 2022 but were destroyed by South African Customs Officials (note that all permits were in place, so the destruction of the seeds was in our opinion unqualified).

In January 2023, two more shipments of seed were sent by CSIRO to UKZN. One batch was sent via registered post (containing 15 accessions of 9 species). This package has still not arrived at UKZN. The second package (containing 24 accessions of 14 species) was sent via courier with appropriate permits attached. This package passed customs, arrived successfully at UKZN, with seeds from 16 accessions belonging to 10 species having successfully germinated (**Table 6**).

Table 5. List of seed accessions sought for nominated non-target host test plant species within the tribe Senecioneae. Species are listed in order of their phylogenetic distance from fireweed.

Species	Accession code	Phylogenetic distance rank	Milestone reporting period	Supplier	Location	State	Collection/ Purchase Date	Additional notes
Senecio madagascariensis	Sen.mad_2	0	1	Ben Gooden	Broughton Vale Road	NSW	19/09/2020	Some seed sent to South Africa March 2021.
Senecio madagascariensis	Sen.mad_3	0	2	Ben Gooden	Bermagui Road-Princes Highway, Tilba Tilba	NSW	23/05/2021	
Senecio madagascariensis	Sen.mad_11	0	1	Ben Gooden	Bogola Headland	NSW	31/12/2020	
Senecio madagascariensis	Sen.mad_12	0	1	Ben Gooden	Central Tilba	NSW	1/01/2021	
Senecio madagascariensis	Sen.mad_13	0	2	Kerinne Harvey	Whiteman Creek	NSW	11/07/2021	
Senecio madagascariensis	Sen.mad_14	0	2	Kerinne Harvey	Koonorigan Road	NSW	10/07/2021	
Senecio madagascariensis	Sen.mad_15	0	2	Michelle Rafter	Wallaces Creek	QLD	10/07/2021	
Senecio madagascariensis	Sen.mad_16	0	2	Michelle Rafter	Wallaces Creek	QLD	10/07/2021	
Senecio madagascariensis	Sen.mad_17	0	2	Michelle Rafter	Yarrabilba	QLD	28/06/2021	
Senecio madagascariensis	Sen.mad_18	0	3	Ben Gooden	Cuttagee Point	NSW	23/07/2022	
Senecio condylus	n/a		n/a	n/a	n/a	n/a	n/a	Multiple Landcare groups in WA contacted, Kings Park contacted. No seed available. Plant is likely to be a recently naturalised plant introduced to WA from South Africa, not an Australian native species.
Senecio glossanthus	n/a	4	n/a	n/a	n/a	n/a	n/a	Seed not available from various SA/WA/vic seed banks.
Senecio spanomerus	Sen.spa_1	7	1	Victorian Conservation Seedbank	Code: JAJ1979	Unknown	Unknown	Fresh seed collected by Peter Jones from CSIRO plants. Some seed sent to South Africa Oct 2021 and July 2022.
Senecio spanomerus	Sen.spa_2	7	1	Kersbrook Landcare Group	Mengerd, Tanunda	SA	11/11/2020	Some seed sent to South Africa July 2022, Jan 2023 and Feb 2023.
Senecio spanomerus (identity uncertain)	Sen.spa_3	7	2	Alice (Swainsona Seed)	Mt Barker Summit	SA	19/01/2022	Species to be confirmed from pressing; Some seed send to South Africa Jan 2023 and Feb 2023.
Senecio brigalowensis	Sen.bri_1	7	2	Bruce McLennan	Wandan	QLD	16/10/2021	Some seed sent to South Africa July 2022, Jan 2023 and Feb 2023.
Senecio brigalowensis	Sen.bri_2	7	2	Bruce McLennan	Grosmont	QLD	15/10/2021	Some seed sent to South Africa July 2022, Jan 2023 and Feb 2023.
Senecio spathulatus	n/a	7	n/a	n/a	n/a	n/a	n/a	Seed not available from various SA/WA/Vic seed banks. Field trip by Caroline Delaisse failed to find any seeds.

Senecio pinnatifolius	Sen.pin_3	7	1	WA Botanic Gardens	Lake Barlee, 5 0km North of Diemal in the Murchison region	WA	Oct-02	Some seed send to South Africa July 2022.
Senecio pinnatifolius (coastal headland variety)	Sen.pin_6	7	3	Ben Gooden	Sawtell NSW	NSW	9/10/2022	
Senecio pinnatifolius (coastal headland variety)	Sen.pin_7	7	3	Ben Gooden	Tacking Point Lighthouse, Port Macquarie	NSW	26/03/2023	
Senecio pinnatifolius var. alpinus	Sen.pin.alp_1	7	1	Ben Gooden	Porcupine trailheading south of Perischer, Kosciuszko National Park	NSW	Feb-19	Some seed sent to South Africa July 2022 and Jan 2023.
Senecio pinnatifolius var. alpinus	Sen.pin.alp_2	7	1	Ben Gooden	Charlotte’s pass	NSW	Jan-19	Some seed sent to South Africa July 2022 and Jan 2023.
Senecio pinnatifolius var. latilobus	Sen.pin.lat_1	7	1	Nindethana Seed Service	Unknown	WA	2018	Some seed sent to South Africa Jan 2023.
Senecio pinnatifolius var. latilobus	Sen.pin.lat_2	7	2	Nindethana Seed Service	Unknown	WA	28/09/2021	Some seed sent to South Africa Jan 2023 and Feb 2023.
Senecio pinnatifolius	Sen.pin.lau_1	7	1	Nindethana Seed Service	Unknown	WA	2018	Some seed sent to South Africa Jan 2023.
Senecio pinnatifolius	Sen.pin.lau_2	7	2	Nindethana Seed Service	Unknown	WA	28/09/2021	Some seed sent to South Africa Feb 2023.
Senecio pinnatifolius var. maritimus	Sen.pin.mar_2	7	2	Nindethana Seed Service	Unknown	WA	28/09/2021	Some seed sent to South Africa Feb 2023.
Senecio pinnatifolius ssp. *Pinnatifolius*	Sen.pin_1	7	1	J. Hoskings	East of Hanging Rock on road to Barry station: 31 31’23’’ S, 151 13’41’’E	NSW	2002	Fresh seeds collected by Peter Jones in quarantine.
Senecio pinnatifolius ssp. *Pinnatifolius*	Sen.pin_4	7	1	Ben Gooden	Handkerchief Beach	NSW	Jul-18	Some seed seed sent to South Africa March 2021, Oct 2021, July 2022 and Jan 2023.
Senecio pinnatifolius var. pinnatifolius	Sen.pin.pin_1	7	2	Ben Gooden	Handkerchief Beach at Nangudga Inlet, Narooma	NSW	23/05/2021	Some seed sent to South Africa Oct 2021, July 2022 and Jan 2023.
Senecio biserratus	n/a	13	n/a	n/a	n/a	n/a	n/a	Not available in SA/VIC seed banks – suggest replacing with *Senecio anethifolius*.
Senecio anethifolius	Sen.ane_1	13	2	Nindethana Seed Service	Unknown	WA	Jul-05	Some seed sent to South Africa Jan 2023.
Senecio anethifolius ssp. Anethifolius	Sen.ane.ane_1	13	2	Unknown	Unknown	Unknown	Unknown	
Senecio anethifolius	Sen.ane_2	13	2	Australian Seed	Unknown	Unknown	27/09/2021	Some seed sent to South Africa Jan 2023.
Senecio distalilobatus	Sen.dis_1	13	1	Ben Gooden	Unknown	Unknown	Jul-18	
Senecio distalilobatus	Sen.dis_2	13	1	Ben Gooden	Captains Flat	NSW	Jan-19	
Senecio bathurstianus	Sen.bat_1	13	1	Ben Gooden	Mount Ainslie	ACT	3/12/2020	
Senecio bathurstianus	Sen.bat_2	13	1	Ben Gooden	Molonglo Gorge	ACT	21/11/2020	Species to be confirmed. Some seed sent to South Africa Feb 2023.
Senecio bathurstianus	Sen.bat_3	13	1	Ben Gooden	Molonglo Gorge	ACT	20/12/2020	

Senecio glomeratus	Sen.glo_1	13	2	Alice (Swainsona Seed)	Lobethal Bushland Park, Reservoir Rd	SA	19/01/2022	Species to be confirmed from pressing. Some seeds sent to South Africa July 2022.
Senecio glomeratus	Sen.glo_2	13	2	Alice (Swainsona Seed)	Lobethal Bushland Park, walking track south of lake	SA	24/01/2022	Species to be confirmed from pressing. Some seed sent to South Africa July 2022 and Jan 2023.
Senecio macrocarpus	Sen.mac_1	13	1	La Trobe University	Saint Albans Rail Reserve	VIC	Unknown	Fresh seeds collected by Peter Jones in quarantine. Some seed sent to South Africa Nov 2020 and July 2022.
Senecio macrocarpus	Sen.mac_2	13	1	Victorian Conservation Seedbank	"JAJ2840", location unknown	VIC	Unknown	Some seed sent to South Africa July 2022.
Senecio linearifolius	Sen.lin_1	13	1	Ben Gooden	Barragga Bay	NSW	29/12/2020	
Senecio linearifolius	Sen.lin_2	13	3	Ben Gooden	Barragga Bay	NSW	3/01/2023	Some seed sent to South Africa Feb 2023.
Senecio linearifolius var. arachnoideus	Sen.lin.ara_2	13	1	Ben Gooden	Big Hole Campground	NSW	Jan-19	Some seed sent to South Africa Feb 2023.
Senecio linearifolius var. arachnoideus	Sen.lin.ara_3	13	2	Ben Gooden	1080 Beach, Mystery Bay	NSW	23/05/2021	Some seed sent to South Africa Oct 2021.
Senecio linearifolius var. latifolius	Sen.lin.lat_1	13	1	Ben Gooden	Gibraltar Rocks, Tidbinbilla	ACT	Jan-19	Some seed sent to South Africa Oct 2021.
Senecio linearifolius var. latifolius	Sen.lin.lat_2	13	3	Ben Gooden	Tidbinbilla	ACT	22/01/2023	
Senecio hispidulus	Sen.his_5	13	2	Ben Gooden, Peter Jones	Endrick River	NSW	Sep-20	
Senecio hispidulus	Sen.his_6	13	2	Ben Gooden	Brou Lake, Bodalla	NSW	23/05/2021	Some seed sent to South Africa Oct 2021, July 2022, and Feb 2023.
Senecio hispidulus (identity uncertain)	Sen.his_7	13	2	Alice (Swainsona Seed)	Corner of Kenton Valley Road and Bretag Rd, at southeastern corner of Lobethal Bushland Park	SA	19/01/2022	Species to be confirmed from pressing.
Senecio hispidulus	Sen.his_8	13	3	Ben Gooden	Wee Jasper	NSW	31/12/2022	Some seed sent to South Africa Feb 2023.
Senecio hispidulus	Sen.his_10	13	1	Ben Gooden	Depot Beach	NSW	23/08/2020	Some seed sent to South Africa July 2022.
Senecio hispidulus	Sen.his_11	13	1	WA Botanic Gardens	Kings Park bushland	WA	Oct-02	
Senecio diaschides	Sen.dia_1	13	1	Ben Gooden	Gibraltar Rocks Tidbinbilla	ACT	Jun-18	Fresh seeds collected by Peter Jones in quarantine. Some seed sent to South Africa July 2022 and Feb 2023.
Senecio diaschides	Sen.dia_2	13	1	Ben Gooden	Tidbinbilla	ACT	26/06/2018	Fresh seeds collected by Peter Jones in quarantine. Some seed sent to South Africa Nov 2020, July 2022 and Feb 2023.
Senecio minimus	Sen.min_1	13	1	LaTrobe Wildlife Sanctuary	Sweeny's Flat Nillumbik	VIC	Mar-16	Some seed sent to South Africa Nov 2020.
Senecio minimus	Sen.min_2	13	1	Ben Gooden	Marble area	NA	Feb-19	Fresh seeds collected by Peter Jones in quarantine March 2020. Some seed sent to South Africa Feb 2023.

Senecio minimus	Sen.min_3	13	1	Tasmania Botanic Gardens	“TSCC0002409”	TAS	NA	
Senecio minimus	Sen.min_4	13	1	Ben Gooden	Neriga Road	NSW	2/01/2020	
Senecio minimus	Sen.min_5	13	2	Ben Gooden	Corunna Point	NSW	2/01/2021	Some seed sent to South Africa Oct 2021 and Feb 2023 (Accesion code previously: Sen.min_4).
Senecio bipinnatisectus	n/a	13	n/a	n/a	n/a	n/a	n/a	Not available in VIC seed banks – suggest replacing with *Senecio picridioides* below,
Senecio picridioides	Sen.pic_1	13	2	Alice (Swansonia Seed)	Lobethal Bushland Park, Reservoir Rd	SA	24/01/2022	
Senecio picridioides	Sen.pic_2	13	2	Alice (Swansonia Seed)	Mt Barker Summit	SA	19/01/2022	
Senecio macranthus	n/a	16	n/a	n/a	n/a	n/a	n/a	Only present in inland NSW; see not sought. Suggest replacing with *Senecio phelleus*.
Senecio phelleus	Sen.phe_1	16	2	Alice (Swansonia Seed)	Lobethal Bushland Park, Reservoir Rd	SA	19/01/2022	Species to be confirmed from pressing. Some seed sent to South Africa Feb 2023.
Senecio gunnii	Sen.gun_1	16	1	Ben Gooden	Rainbow lake	NSW	Jan-19	Fresh seeds collected by Peter Jones in quarantine Jan 2020. Some seed sent to South Africa Feb 2023.
Senecio gunnii	Sen.gun_2	16	1	Ben Gooden	Charlotte’s pass	NSW	Jan-19	Fresh seeds collected by Peter Jones in quarantine Jan 2020. Some seed sent to South Africa Nov 2020 and Feb 2023.
Senecio prenanthoides	Sen.pre_1	16	1	Ben Gooden	Mount Ainslie	ACT	8/11/2020	Species to be confirmed. Some seed sent to South Africa Feb 2023.
Senecio prenanthoides	Sen.pre_2	16	1	Ben Gooden	Molonglo Gorge	NSW	7/07/2020	Species to be confirmed. Some seed sent to South Africa Feb 2023.
Senecio magnificus	Sen.mag_1	16	1	Nindethana Seed Service	NA	WA	Jul-18	Some seed sent to South Africa Nov 2020.
Senecio magnificus	Sen.mag_2	16	1	PlantBank RBG	White Cliffs	NSW	Nov-00	Some seed sent to South Africa Nov 2020.
Senecio magnificus	Sen.mag_3	16	1	ANBG?	CAN868413			
Senecio quadridentatus	Sen.qua_5	16	2	Alice (Swansonia Seed)	Lobethal Bushland Park, Reservoir Rd	SA	19/01/2022	Species to be confirmed from pressing. Some seed sent to South Africa Feb 2023.
Senecio quadridentatus	Sen.qua_6	16	2	Alice (Swansonia Seed)	Corner of Kenton Valley Road and Bretag Rd, at south eastern corner of Lobethal Bushland Park	SA	19/01/2022	Species to be confirmed from pressing.
Senecio quadridentatus	Sen.qua_7	16	2	Alice (Swansonia Seed)	Lobethal Bushland park, walking track west of lake	SA	24/01/2022	Species to be confirmed from pressing.
Senecio quadridentatus	Sen.qua_8	16	2	Alice (Swansonia Seed)	Lobethal Bushland Park, near lake on the western side	SA	24/01/2022	Species to be confirmed from pressing.
Senecio quadridentatus	Sen.qua_9	16	3	Ben Gooden	Tidbinbilla	ACT	22/01/2023	Some seed sent to South Africa Feb 2023.

Senecio quadridentatus	Sen.qua_11	16	1	Ben Gooden	Darlington point, Murrumbidgee River near Griffith.	NSW	11/11/2020	Some seed sent to South Africa Feb 2023.
Senecio quadridentatus	Sen.qua_12	16	1	WA Botanic Gardens	15 km on Peak Charles Road, Peak Charles National Park, southern goldfields/mallee region	WA	Unknown	
Senecio queenslandicus	n/a	16	n/a	n/a	n/a	n/a	n/a	Infrequently sighted on ALA, and seed not available.
Arrhenechthites mixtus	Arr.mix_1	19	1	PlantBank RBG	Glow worm tunnels road, Central Highlands	QLD	Dec-07	
Senecio vagus	n/a	19	n/a	n/a	n/a	n/a	n/a	Not available from VIC seed banks. Populations sought in NSW but no seed detected.
Senecio velleioides	Sen.vel_1	23	1	PlantBank RBG	Mumbulla Trig	NSW	1/11/2007	Some seed sent to South Africa Nov 2020.
Senecio velleioides	Sen.vel_3	23	2	Ben Gooden	Carter Creek crossing on Western Distributor Road, Currowan	NSW	30/01/2022	
Bedfordia arborescens	Bed.arb_1	27	1	Seeds of Gippsland	Strzelecki Ranges	VIC	Jan-18	

4.3 Host-specificity trials undertaken at UKZN laboratories in South Africa

Host-specificity methods

Host-specificity trials were conducted in the insectary at the University of KwaZulu-Natal's Pietermaritzburg Agriculture Campus, South Africa, at an average temperate of 24 °C, ambient relative humidity and 12:12 hr day:night cycle. For each trial, one mating weevil pair was exposed to a single potted (18 cm diameter pot) non-target test plant, housed within a 69 cm plastic sleeve. Plants were observed for oviposition scars, with each scar tagged to enable easy detection of where the larval damage/feeding might occur.

After 4 days, plants were removed from the laboratory and moved to a shade house in the University's Botanical Garden. A single control fireweed plant was exposed to the same mating weevil pair for 4 days after they had first been exposed to the non-target plant (as such, a single trial consisted of one mating weevil pair being exposed first to the non-target plant and then to the single fireweed plant). The weevils were then returned to the lab-reared culture.

Plants were monitored weekly in the shade house for signs of external larval feeding damage, such as frass production. After 66 days, plants were returned to the laboratory and kept under grow lights in BugDorm cages. Plants were then checked daily for the emergence of adult weevils. After 80 days, plants were dissected to inspect internal larval feeding damage. The length of boring damage along stems was measured as well as the presence and size of any larvae and pupae found within the stems. The list of species deployed to date in host specificity trials at UKZN, and a summary of oviposition, larval development and feeding data, are provided in **Table 7**.

Host-specificity results

Host-specificity trials have been completed on at least one accession of each of four Australian non-target species: *S. hispidulus, S. diaschides, S. quadridentatus* and *S. linearifolius* (three replicates per accession, see **Table 7**). Weevils oviposited on three of these non-target species (*S. hispidulus, S. diaschides* and *S. quadridentatus*), but not on *S. linearifolius*. The number of eggs laid was significantly higher on fireweed plants (mean ± SE = 9.0 ± 1.00 eggs) than on *S. hispidulus* (2 ± 0.58 eggs), S. diaschides (1.3 ± 0.89) or *S. quadridentatus* (3 ± 1.73 eggs). After 14 weeks, no adults had emerged from any of the test plants; they were subsequently dissected to determine if eggs had hatched, and larvae developed.

A mean of 1.7 larvae per plant was detected in the fireweed control plants. No larvae were detected in the stems of *S. hispidulus*, whilst a mean of 2.3 larvae per plant was recorded in the stems of *S. quadridentatus*. In total, 12 larvae were found but only one of these, recovered from a fireweed stem, was still alive at the time of plant dissection. An average of 0.7 pupae were found in the fireweed control plants but none were found in any of the Australian native *Senecio* species. The length of stem-boring damage was lower in the Australian native *Senecio* species compared to the fireweed controls, with a mean of 61.7

cm bored on fireweed and 16.3 cm on *S. quadridentatus*, with no stem-boring detected in *S. hispidulus*. Data for *S. diaschides* are still being collected and could not be included in this report. These results indicated that, under laboratory conditions at UKZN, the weevil is able to readily oviposit on phylogenetically distantly related native Australian *Senecio* species and undergo limited larval development and damage to non-target host plant stems, but not able to complete development to pupation stage.

Additional host-specificity trials have been initiated for two native Australian species (*S. hispidulus* and *S. diaschides*) and several native South African plant species within the tribe Senecioneae (e.g., *Euryops chrysanthemoides, Kleinia fulgens,* etc., see **Table 7**). These native South African plants were added to the test list either for their horticultural importance in Australia, or because they co-occur with fireweed across its native South African range but on which *Gasteroclisus* larvae have never been detected during host range surveys. Including co-occurring native South African plant species will enable us to test if the weevil utilises non-target plant species differently under laboratory compared to field conditions. Weevils oviposited on all fireweed control plants, the two native Australian species (*S. hispidulus* and *S. diaschides*), the field collected species (*S. polyanthemoides*), and two of the six horticultural plants tested (**Table 7**). The mean number of eggs laid per plant was higher on fireweed (4.9 ± 0.19) than on any of the other non-target plant species. To date, larvae and larval feeding damage were only recorded on *Euryops chrysanthemoides* and *S. polyanthemoides*.

Prospects for host-specificity testing at UKZN in South Africa

Given the problems associated with the growing of the test plants (e.g., delays in obtaining seeds from Australia) and COVID-19 pandemic in 2020 through to early 2022, work on the host specificity of *G. tricostalis* was initially delayed but will continue at UKZN beyond the end date of this project. Specifically, these trials will include both no-choice and choice tests on selected South African (notably those surveyed in the field) and Australian species of *Senecio*. These studies will form the final chapter of Dineshen Singh's PhD thesis and will supplement the work that he has already completed on the weevil's field host range and impact on the growth and reproduction of fireweed. This is scheduled for completion at the end of 2023, when submission of his thesis is due.

Table 6. Germination of seeds of Australian native *Senecio* species sent to UKZN laboratories in South Africa, January 2023.

Species	Phylogenetic distance rank	Code	No. of germinants
S. brigalowensis	7	(Sen.bri-1)	5
S. brigalowensis	7	(Sen.bri-2)	4
S. pinnatifolius var latilobus	7	(Sen.pin.lat-2)	0
S. pinnatifolius	7	(Sen.pin.lau-2)	0
S. pinnatifolius var maritimus	7	(Sen.pin.mar-2	0
S. spanomerus	7	(Sen.spa-2)	0
S. spanomerus	7	(Sen.spa-3)	Numerous
S. bathurstianus	13	(Sen.bat-1)	Numerous
S. hispidulus	13	(Sen.his-6)	1

S. hispidulus	13	(Sen.his-8)	1
S. diaschides	13	(Sen.dia-1)	Numerous
S. diaschides	13	(Sen.dia-2)	Numerous
S. minimus	13	(Sen.min-2)	5
S. minimus	13	(Sen.min-5)	Numerous
S. linearifolius	13	(Sen.lin-2)	10
S. linearifolius	13	(Sen.lin.ara-2)	0
S. gunnii	16	(Sen.gunnii-1)	0
S. gunnii	16	(Sen.gunnii-2)	0
S. phelleus	16	(S.phe-1)	0
S. prenanthoides	16	(Sen.pre-1)	0
S. prenanthoides	16	(Sen.pre-2)	1
S. quadridentatus	16	(Sen.quad-5)	2
S. quadridentatus	16	(Sen.quad-9)	0
S. quadridentatus	16	(Sen.quad-11)	0

Table 7. Summary of host-specificity test results for trials undertaken at UKZN laboratories in South Africa.

Species	Accession	Mean (± SD) No. eggs	Mean (± SD) No. Larvae	Mean (± SD) No. Pupae	Mean (± SD) Larvae tunnel length (cm)	Species group	Phylogenetic distance rank	Reporting period*
Host specificity testing undertaken at UKZN								
Senecio madagascariensis	S. mad	9.0 (± 1.00)	1.7 (± 0.88)	0.7 (± 0.67)	61.7 (± 13.38)	Control	0	2
Senecio hispidulus	Sen. Hisp-4	2.0 (± 0.58)	0.0 (± 0.00)	0.0 (± 0.00)	0.0 (± 0.00)	Australian native	13	2
Senecio linearifolius	Sen. Lin. Ara -3	0.0 (± 0.00)	0.0 (± 0.00)	0.0 (± 0.00)	0.0 (± 0.00)	Australian native	13	2
Senecio quadridentatus	Sen. Quad-10	3.0 (± 1.73)	2.3 (± 1.20)	0.0 (± 0.00)	16.3 (± 8.57)	Australian native	16	2
Senecio hispidulus	Sen. Hisp-6	0.5 (± 0.50)	0.5 (± 0.50)	0.5 (± 0.71)	0.0 (± 0.00)	Australian native	13	3
Senecio diaschides	Sen. Dia-2	1.3 (± 0.89)	1.3 (± 0.89)	Ongoing	Ongoing	Australian native	13	3
Senecio polyanthemoides	S. poly-1	1.0 (± 0.58)	1.0 (± 0.58)	1.0 (± 1.00)	0.0 (± 0.00)	Native South African *Senecio* collected from the field	Unknown	3
Euryops chrysanthemoides	E. chry-1	1.0 (± 0.58)	1.0 (± 0.58)	1.0 (± 1.00)	0.0 (± 0.00)	Horticultural	24	3
Kleinia fulgens	K. fulg-1	1.0 (± 1.73)	1.0 (± 1.73)	Ongoing	Ongoing	Horticultural	23	3
Kleinia galpinii	K. galpi-1	0.0 (± 0.00)	0.0 (± 0.00)	Ongoing	Ongoing	Horticultural	23	3
Senecio ficoides	S. fic-1	0.0 (± 0.00)	0.0 (± 0.00)	0.0 (± 0.00)	0.0 (± 0.00)	Horticultural	23	3
Senecio talinoides	S. tal-1	1.0 (± 1.00)	1.0 (± 1.00)	Ongoing	Ongoing	Horticultural	23	3
Senecio tamoides	S. tam-1	0.3 (± 0.33)	0.3 (± 0.33)	0.0 (± 0.00)	0.0 (± 0.00)	Horticultural	23	3
Senecio bupleuroides	S. bup-1	1.0 (± 1.00)	Ongoing	Ongoing	Ongoing	Native South African *Senecio* collected from the field	Unknown	3
Senecio retrorsus	S. ret-1	1.3 (± 1.15)	Ongoing	Ongoing	Ongoing	Native South African *Senecio* collected from the field	Unknown	3
Senecio madagascariensis	S. mad	4.6 (± 2.39)	3.2 (± 1.59)	0.0 (± 0.00)	46.3 (± 23.81)	Control	0	3

4.4 Host-specificity trials undertaken at CSIRO laboratories in Australia

Preliminary host-specificity testing undertaken in 2021

Commencing on 17th June 2021, we undertook a preliminary host-specificity trial within the Australian quarantine facility by exposing the highly fecund weevil pair from consignment BAA385917 to four non-target native *Senecio* species within a single cage (i.e., multi-choice, weed-absent design): *S. pinnatifolius* var. *alpinus*, *S. pinnatifolius* var. *pinnatifolius*, *S. spanomerus*, *S. diaschides* (**Figure 19**). The fireweed plants on which the weevil pair had already oviposited acted as experimental controls. The weevils were maintained in the cage with the four non-target *Senecio* species for 7 days before being returned to the main weevil culture on the fireweed host plants. The aim of this preliminary trial was to begin optimising host-specificity methods for deployment in future tests, and to capitalise on the opportunity afforded to us by the possession of a highly fecund female weevil and four very healthy non-target plant species, three of which are relatively closely related to fireweed (*S. pinnatifolius* and *S. spanomerus*).

On 17th June 2021, at the time when the weevil pair was removed from the cage and returned to the main weevil culture, two oviposition scars were detected on the *S. pinnatifolius* var. *alpinus* plant (**Figure 20a**). Oviposition was not observed on the other three non-target plants located in the same cage. Adult leaf feeding damage was not observed on any plant.

By 25th June 2021, the apical stem tip above each oviposition scar on the *S. pinnatifolius* var. *alpinus* plant had begun to wither, and by 5th July the stems with oviposition scars had begun to darken, shrivel and completely collapse (**Figure 20b**). By 5th July, the other stems (without oviposition scars) on the *S. pinnatifolius* var. *alpinus* plant had begun to wilt but were still green, fleshy and without evidence of weevil feeding damage. On 25th June, we also observed characteristic darkening and wilting of a single small stem on the *S. pinnatifolius* var. *pinnatifolius* plant (**Figure 20c**), but this was not accompanied by any evidence of oviposition, so may have been coincidental to weevil attack.

The trial was terminated on 5th July 2021 due to complete collapse and rapid deterioration of the *S. pinnatifolius* var. *alpinus* plant, at which time all plants were removed from pots and carefully dissected. Although larval feeding damage was evident on the *S. pinnatifolius* var. *alpinus* stems, we were unable to detect any frass (likely due to very dark and deteriorated stem tissue) or the larvae themselves, meaning we do not have definitive evidence for larval emergence, feeding and development at the sites of oviposition. The larvae, if they did indeed incur damage to the stems, likely died when the stems began to deteriorate and decomposed by the time the stems were dissected. All other plants remained green, robust, fleshy, and healthy over the course of the trial (except for the single small stem on *S. pinnatifolius* var. *pinnatifolius* above the oviposition scar).

Figure 19. Native *Senecio* species used in a single multi-choice host-specificity trial under quarantine conditions in Australia, commencing 10th June 2021; (a) *S. pinnatifolius* var. *pinnatifolius*, (b) *S. pinnatifolius* var. *alpinus*, (c) *S. spanomerus*, (d) *S. diaschides*.

Figure 20. Weevil damage to a single *S. pinnatifolius* var. *alpinus* plant in the preliminary multi-choice host-specificity trial under quarantine conditions in Australia, commencing 10th June 2021: (a) oviposition scar (yellow arrow) and (b) withered stem around where the two weevil eggs were laid. (c) Possible larval feeding damage to *S. pinnatifolius* var. *pinnatifolius* stem (yellow arrow).

Methods for host-specificity trials commenced at CSIRO in 2023

To date, 11 Australian native *Senecio* species have been reared under quarantine conditions at the CSIRO laboratories in Canberra for deployment in host-specificity trials. Preference was given to native species within the phylogenetic distance clade number 7, which represents the most closely related group of plant species to fireweed in Australia (including *S. pinnatifolius*, *S. spanomerus* etc).

Oviposition and larval development host-specificity trials were undertaken using a "no-choice/host added" design (modified from Balciunas et al. 2010). For each trial, three mating pairs of F1 adult weevils (drawn from the lab-reared culture) were exposed to three different non-target plant species in a cage measuring 40 cm X 40 cm X 60 cm (see **Table 8** for list of plant species deployed in each trial). The weevils selected for deployment in each host-specificity trial were reproductively viable, having been observed to have oviposited previously on fireweed host plants within the lab-reared culture.

It is important to note that the weevils deployed in these host-specificity trials had been reared on their preferred host plant species (fireweed), and then maintained on fireweed plants upon adult emergence until they had reached reproductive maturity. As such, their interactions with the non-target plant species must be evaluated in the context of pre-conditioning to the target weed. In some weed biocontrol systems, pre-conditioning of insects to their preferred host can result in biases against oviposition on non-target plant species. However, for the fireweed-weevil biocontrol system, it was deemed to be not biologically feasible to undertake oviposition choice and larval development host-specificity trials using naïve adult weevils, given that larvae must first develop through to adulthood on fireweed, and adults must also feed on fireweed to develop healthy gonads and reach reproductive maturity. In this context, the aim of these host-specificity trials was to simply test whether there is a risk of weevils reared on fireweed 'spilling over' on to non-target host plants in situations where their preferred host plant (fireweed) is not available for oviposition, or where the two species co-occur at a site. As such, these trials represent conservative and ecologically relevant tests of non-target damage, as in a field context, there is always likely to be populations of fireweed intermixed with other native *Senecio* species.

NB – physiological host ranges of naïve weevils may be tested in future for some high-priority native species (e.g., S*enecio pinnatifolius*) by transferring eggs laid on fireweed to the non-target plant species, then tracking larval emergence, feeding and development through to adult emergence. Such tests bypass adult female oviposition choice and are thus deemed highly conservative and, in some cases, ecologically unrepresentative for use in host-specificity testing. To date, there has been no need to undertake egg transfers, because female weevils have been found to readily oviposit on the non-target plant species (see results below).

During the period of exposure to the weevils, the non-target test plants were observed for feeding damage by the adult weevils (i.e., number of leaves and percentage leaf surface area damaged per plant) and number of oviposition scars, which were individually tagged to enable monitoring of larval feeding damage over time. We also recorded plant size (height, and number of lateral stems; data not presented in this report). All plants were reproductively mature at the time of exposure to the weevils.

After 4 days, the three non-target plants were removed from the cage and monitored weekly thereafter for signs of larval damage. Photographs were taken of each oviposition scar and visible evidence of larval feeding damage, including accumulation of frass. On the same day that the three non-target plants were removed, a single healthy and reproductively mature fireweed plant was added to each cage for exposure to the same set of weevils for a further 4 days. The fireweed plant acted as an experimental control, such that a trial was only considered valid if the weevils oviposited on the fireweed plant, followed by larval emergence, feeding and development over multiple instar stages to pupation (similar rationale to the UKZN host-specificity experiments).

After 4 days, the fireweed plant was transferred to the same cubicle containing the three non-target plants and monitored for signs of larval development. Plants were maintained at an average temperate of 23.8 °C, relative humidity of 64.6 %, and LED lights set at 4,000 Kelvin (equivalent to natural sunlight) at a 14:10 hr day:night cycle. The three weevil pairs were transferred to a new cage containing a separate fireweed plant for ongoing rearing. All weevils deployed in each host-specificity trial were kept separate from the original culture and never utilised in further tests, to improve the statistical independence amongst trials.

After 80 days, all plants will be carefully dissected to record the presence of larval damage within each stem (focussing on areas below each tagged oviposition scar) and, where larvae were detected, the length of larval feeding tunnels along each stem. The number and size (length, cm) of larvae and pupae will be recorded, then carefully transferred to the stems of healthy plants for ongoing rearing to adulthood. The plants will be dissected at 80 days because the average time to adult emergence under the temperature, humidity and light conditions stated above at the CSIRO laboratories is 76 days. However, plants will be dissected earlier than 80 days if they exhibit signs of senescence.

NB – data presented in this report (below) were obtained from plants in trials 1 and 2, and a single plant from trial 4, which showed signs of severe damage and were dissected after 65 days. All other plants have data for oviposition and adult weevil feeding damage but not for larval damage, pupation or adult emergence, as the trials are still ongoing.

Results of host-specificity trials commenced at CSIRO in 2023

To date, eight multi-species host-specificity trials have been initiated for seven of the 11 Australian native *Senecio* species. Weevils were observed to oviposit on six of these native *Senecio* species, including several species that are relatively distantly related to fireweed (e.g., *S. hispidulus*, *S. prenanthoides*). However, a linear regression analysis revealed that the density of oviposition scars declined significantly with increasing phylogenetic distance of non-target plant species from fireweed (R^2 = 0.2974, F = 10.5836, P = 0.0033, **Figure 21**), with the relatively more closely related species (*S. pinnatifolius*, *S. spanomerus* etc) being most at risk of high rates of oviposition. Feeding by adult weevils on non-target and fireweed leaf tissue was low and had negligible effects on plant health.

At 65 days following exposure to the adult weevils, plants from Trials 1 and 2 were dissected and examined for larvae, signs of larval feeding damage and pupation. Trial 1 was broken down because rates of oviposition were generally very low and all plants remained healthy with no signs of larval feeding damage – hence we suspected that either no or very few larvae would be present, and none were developing across multiple instar stages to pupation and adult emergence. This was proved to be correct, with only minimal larval feeding damage detected on the control fireweed plant and no larvae detected within any plant, including the control.

Trial 2 was broken down at 65 days because most plants were showing signs of severe larval feeding damage, stem collapse and in two cases (*S. hispidulus* and the control fireweed plant) complete senescence (depicted in **Figure 22**). Adult weevils had oviposited on all plants, including the three non-target native *Senecio* species. Larval feeding damage was, unexpectedly, generally more extensive on the non-target species (especially *S. hispidulus*) than the control fireweed plant. F1 adults had emerged on two of the non-target plants (one each on *S. hispidulus* and *S. spanomerus*) and the control fireweed plant (3 x F1 adults).

Prospects of ongoing host-specificity testing for *Gasteroclisus tricostalis*

The results from Trial 2 (albeit non-replicated at this stage) indicate that, at least under laboratory conditions, the weevil *G. tricostalis* is able to develop through to emergence of F1 adults on some native Australian *Senecio* species that are relatively distantly related to fireweed. Further research will be required to ascertain the broader risk of non-target damage and adult development on native species, which will likely consist of:

- Completing no-choice testing for multiple replicates for the 10 priority *Senecio* species currently maintained under quarantine conditions at CSIRO laboratories in Canberra, using the same methods as described above and presented in **Table 8**. Although the current project formally closes on 2nd June 2023, both CSIRO and UKZN researchers will sustain the host-specificity research until at least the end of the 2023 calendar year. Further investment would be required to support host-specificity trials of more than 10 non-target species at the CSIRO laboratories in Canberra.
- Undertaking continuation trials to see if the F1 adults that emerge from the non-target host plants (in this case, at least for *S. hispidulus* and *S. spanomerus*) can be sustained over multiple generations on those same non-target plant species (**Figure 23**). This research has already commenced for the adults that emerged on *S. hispidulus* and *S. spanomerus*.
- Undertaking multi-choice trials, where reproductively mature adult weevils are exposed to the non-target plant species in the context of a co-occurring fireweed plant (**Figure 23**). These multi-choice trials represent more ecologically relevant tests of female oviposition preference that would be expected to occur under field conditions where fireweed and other native *Senecio* species co-occur in the Australian environment. Further investment would be required to support multi-choice experimentation for species at high risk of non-target damage.

Table 8. Results of host-specificity trials for seven Australian native *Senecio* species at CSIRO laboratories in Canberra (as of 2nd June 2023). Many of the replicate plants species highlighted in grey were observed to be significantly damaged (presumably by weevil attack) and were dissected on 24th May 2023 (i.e., 65 days after first being exposed), 15 days earlier than expected. A description of damage is provided as a footnote to the table, and presented for plant specimens deployed in Trial No. 2 in Figure 22.

Trial number	Test species	Phylogenetic distance rank	Accession code	Accession replicate number	Host-test commencement date (exposed to weevils)	Weevil damage*					
						% Leaf damage by adult weevils	Number of oviposition scars	Larvae No.	Larval tunnelling damage (sum cm)	Pupae No.	Adult No.
1	*Senecio spanomerus*	7	Sen.spa-2	1	20/03/2023	1	3	0	0	0	0
1	*Senecio quadridentatus*	16	Sen.quad-11	1	20/03/2023	0	0	0	0	0	0
1	*Senecio hispidulus*	13	Sen.his-2	1	20/03/2023	1	0	0	0	0	0
1	*Senecio madagascariensis*	0	Sen.mad-10	1	24/03/2023	5	4	0	3	0	0
2	*Senecio spanomerus*	7	Sen.spa-1	1	20/03/2023	0	4	0	8	0	1
2	*Senecio hispidulus*	13	Sen.his-2	2	20/03/2023	1	4	0	24	2	1
2	*Senecio hispidulus*	13	Sen.his-2	3	20/03/2023	0	3	1	11	0	0
2	*Senecio madagascariensis*	0	Sen.mad-10	2	24/03/2023	5	10	0	8	0	4
3	*Senecio pinnatifolius*	7	Sen.pin-4	1	4/04/2023	5	0	n/a	n/a	n/a	n/a
3	*Senecio spanomerus*	7	Sen.spa-1	2	4/04/2023	0	0	n/a	n/a	n/a	n/a
3	*Senecio hispidulus*	13	Sen.his-3	1	4/04/2023	1	0	n/a	n/a	n/a	n/a
3	*Senecio madagascariensis*	0	Sen.mad-10	3	8/04/2023	5	5	n/a	n/a	n/a	n/a
4	*Senecio hispidulus*	13	Sen.his-2	4	13/04/2023	0	3	n/a	n/a	n/a	n/a
4	*Senecio spanomerus*	7	Sen.spa-1	3	13/04/2023	1	8	1	5	0	0
4	*Senecio pinnatifolius*	7	Sen.pin-4	2	13/04/2023	1	4	n/a	n/a	n/a	n/a
4	*Senecio madagascariensis*	0	Sen.mad-13	4	17/04/2023	5	12	n/a	n/a	n/a	n/a
5	*Senecio diaschides*	13	Sen.dia-1	1	21/04/2023	0	0	n/a	n/a	n/a	n/a
5	*Senecio prenanthoides*	16	Sen.pre-3	1	21/04/2023	1	0	n/a	n/a	n/a	n/a
5	*Senecio pinnatifolius*	7	Sen.pin-1	2	21/04/2023	1	0	n/a	n/a	n/a	n/a
5	*Senecio madagascariensis*	0	Sen.mad-10	5	25/04/2023	5	5	n/a	n/a	n/a	n/a
6	*Senecio pinnatifolius*	7	Sen.pin-1	3	28/04/2023	2	4	n/a	n/a	n/a	n/a

6	*Senecio brigalowensis*	7	Sen.bri-1	1	28/04/2023	0	2	n/a	n/a	n/a	n/a
6	*Senecio prenanthoides*	16	Sen.pre-3	2	28/04/2023	5	3	n/a	n/a	n/a	n/a
6	*Senecio madagascariensis*	0	Sen.mad-13	6	18/05/2023	5	2	n/a	n/a	n/a	n/a
7	*Senecio pinnatifolius*	7	Sen.pin-1	3	25/05/2023	1	3	n/a	n/a	n/a	n/a
7	*Senecio brigalowensis*	7	Sen.bri-1	2	25/05/2023	3	10	n/a	n/a	n/a	n/a
7	*Senecio spanomerus*	7	Sen.spa-2	2	25/05/2023	0	3	n/a	n/a	n/a	n/a
7	*Senecio madagascariensis*	0	Sen.mad-16	1	29/05/2023	n/a	n/a	n/a	n/a	n/a	n/a
8	*Senecio brigalowensis*	7	Sen.bri-1	2	30/05/2023	n/a	n/a	n/a	n/a	n/a	n/a
8	*Senecio prenanthoides*	16	Sen.pre-3	3	30/05/2023	n/a	n/a	n/a	n/a	n/a	n/a
8	*Senecio diaschides*	13	Sen.dia-2	1	30/05/2023	n/a	n/a	n/a	n/a	n/a	n/a
8	*Senecio madagascariensis*	0	Sen.mad-16	2	3/06/2023	n/a	n/a	n/a	n/a	n/a	n/a

* **Trial 1:** Minimal larval damage to all plants, including the fireweed control plant. **Trial 2**: Sen.spa-1 rep 1, no overall plant growth after 65 days (about 9 weeks), with death of at least 50 % of stems on which weevils oviposited; plant supported development of a single F1 adult weevil. Sen.his-2 rep 2, no overall plant growth after 65 days (about 9 weeks); no significant damage to plant stems despite extensive larval tunnelling of stems and emergence of a single F1 adult weevil. Sen.his-2 rep 3, complete plant death as a result of larval damage to the basal stem at the intersection with the root crown. Sen.mad-10 rep 2, complete plant death and emergence of four F1 adult weevils. **Trial 4**: Sen.spa-1 rep 3, complete plant death as a result of larval damage to the basal stem at the intersection with the root crown.

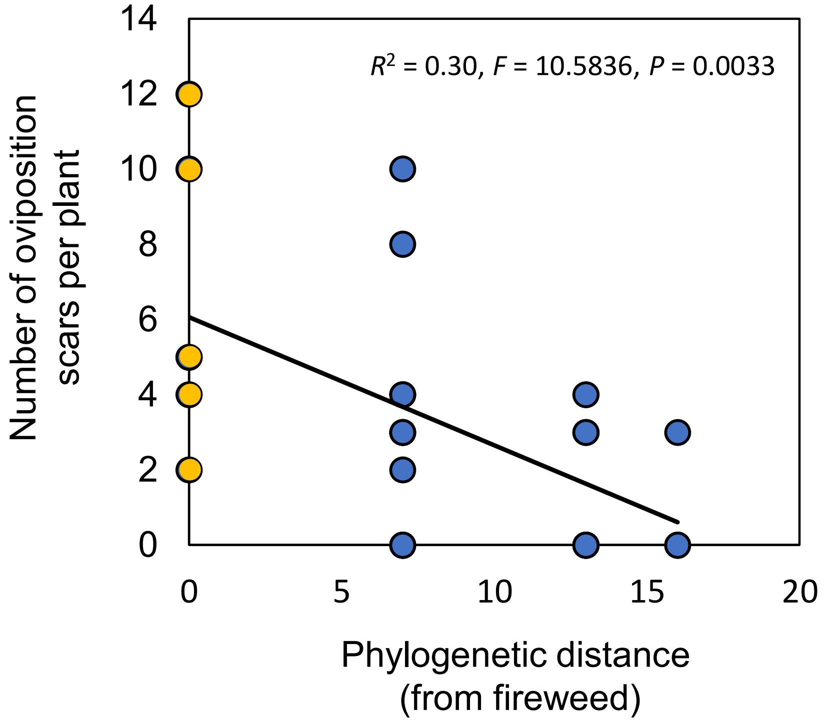


Figure 21. Linear regression analysis of the association between phylogenetic distance of each non-target *Senecio* species (blue points) from fireweed (orange points) and the number of oviposition scars detected on each replicate plant. The black line represents the line-of-best-fit.

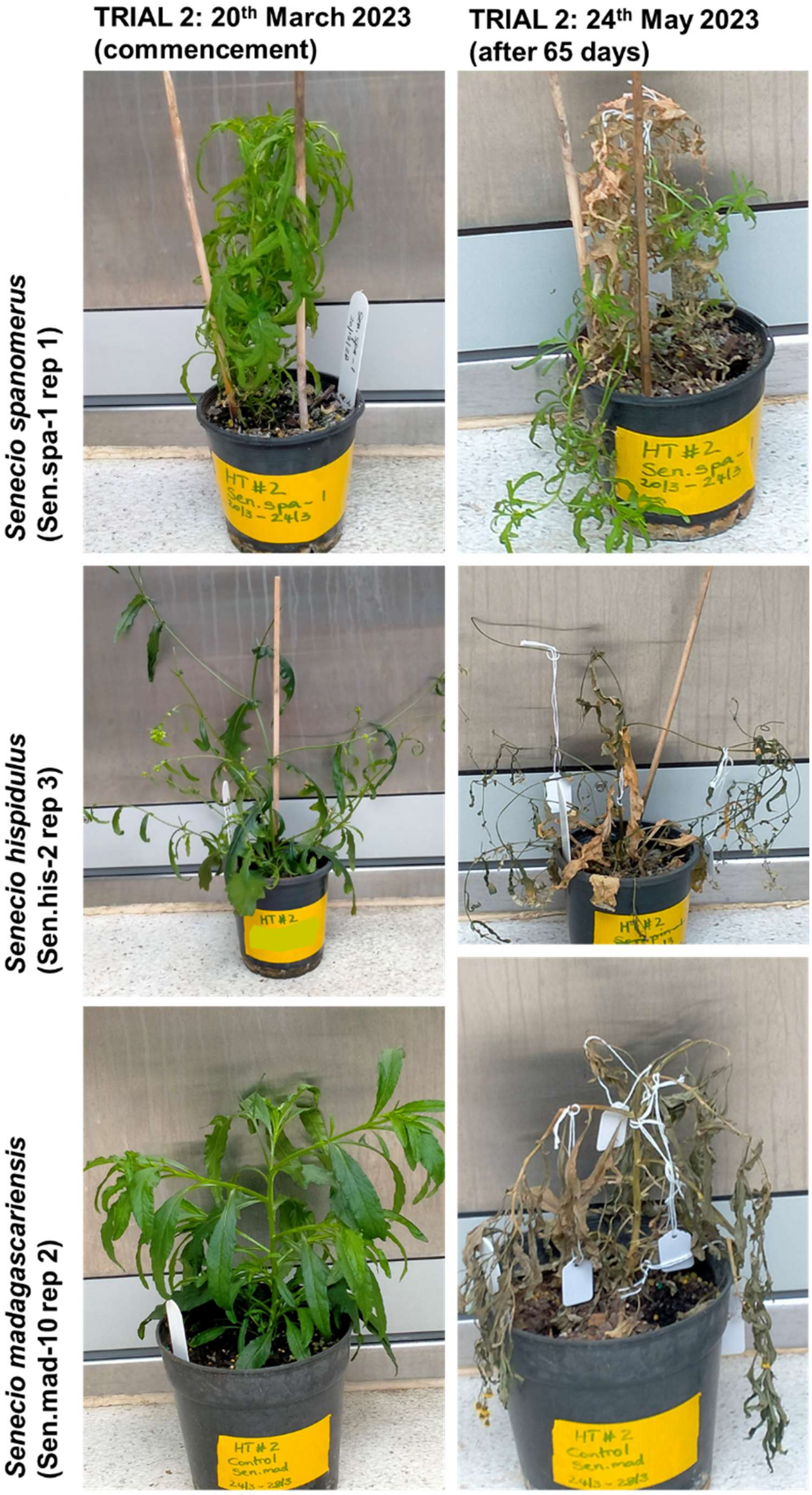


Figure 22. ***Senecio*** **specimens deployed in host-specificity trial number 2, showing signs of severe stem damage as a result of infestation by** ***Gasteroclisus tricostalis*** **weevil larvae .**

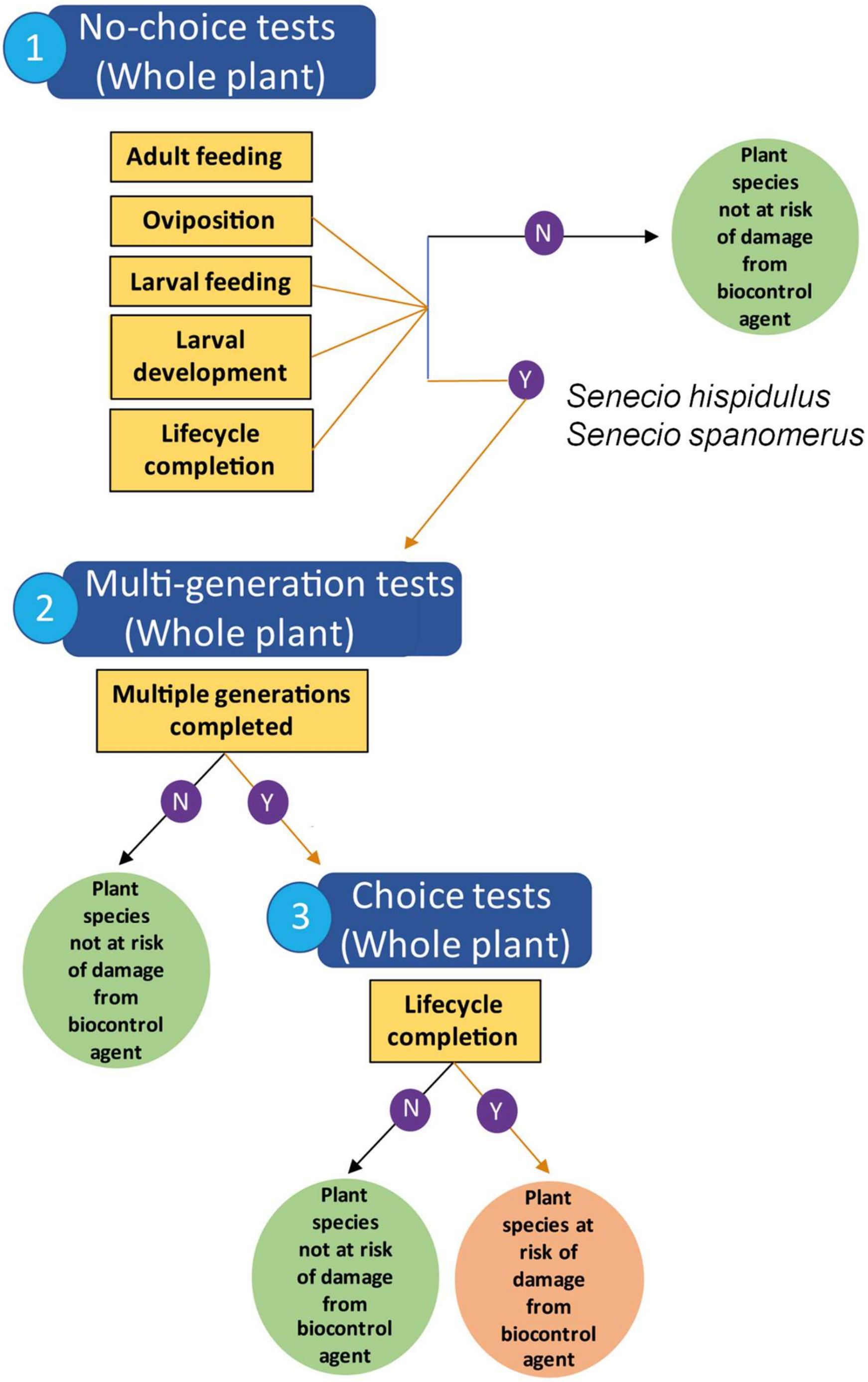


Figure 23. Decision tree used to determine the types of future host-specificity tests to be undertaken for *Gasteroclisus tricostalis* for the target species, fireweed (*Senecio madagascariensis*). Figure has been adapted from a decision for biocontrol research on African boxthorn developed by CSIRO researcher Dr Michelle Rafter.

4.5 Revising the native field host range of stem-boring weevils (with a focus on *Gasteroclisus tricostalis*) across South Africa using phylogenetic analysis

Methods

To further refine the field host range of stem-boring weevils, with a focus on the priority weevil *G. tricostalis*, the data from a PhD (2014-2015, Egli *et al.* 2020) and an MSc project (2017-2018, Singh 2019) were united into a single new/updated phylogenetic analysis. Across both studies, a total of 22 *Senecio* species were surveyed at 34 sites across South Africa (**Table 9**). At each site, either 5 (2017-2018) or 10 (2014-2015) plants per species were uprooted and placed into brown bags and frozen for later dissection. During the 2017-2018 surveys, 10 sweep net samples were taken at each site from each species. Each sweep net sample involved sweeping 10 plants three times each and emptying the contents into a Ziplock bag, which was taken to the laboratory and frozen. At each site, two extra plants were collected; one was pressed and lodged in the John Bews Herbarium and the other was used to identify the species using the key in *Compositae in Natal* (1977).

The frozen plants were inspected for any adult weevils present on the external surface of stems. Adult weevils were removed and stored in 100 % ethanol. The stems of the plants were then dissected under a light microscope and stem-boring larvae were removed and stored in 100 % ethanol. The contents of the Ziplock bags were searched and adult weevils were removed and also preserved in 100 % ethanol. Weevil samples collected on fireweed during the 2017-2018 seasonal surveys (undertaken by Singh *et al*. 2022) were also included in the analysis). The adults were identified as far as possible and then the DNA of the specimens was extracted, and the COI gene sequenced. These DNA sequences were analysed, and a phylogeny was produced (**Figure 24**). The sequences were then sorted into Species Clusters to enable the identification of unique taxa.

Results

Combined across the two datasets, a total of 192 individual insect specimens were sequenced (59 from 2014-2015 and 133 from 2017-2018) from 14 host *Senecio* species. These sequences formed a total of 19 Species Clusters (**Figure 24**). The largest cluster contained sequences of *Gasteroclisus tricostalis* (96 sequences). Previous analyses including fewer sequences found that *G. tricostalis* belonged to either two (Singh 2019) or three (Egli *et al*. 2020) species clusters – indicating *G. tricostalis* to be a species complex rather than a single clade. However, the revised phylogeny presented here, based on the combination of the two previous datasets, was significantly more powerful in terms of breadth of sequence replication over multiple years, growing seasons and sites, and has revealed *G. tricostalis* likely forms a single species cluster. This is consistent with morphological analysis that showed there is only a single species with no morphological variation amongst genitalia or other diagnostic structures.

The sequences belonging to *G. tricostalis* were collected on four plant species: *S. madagascariensis, S. harveianus, S. inaequidens,* and *S. skirrhodon*. These four *Senecio* species form part of the fireweed species complex and are much more closely related to one other than they are to the Australian native *Senecio* species (Schmidt-Lebuhn et al. 2022). This revised phylogeny therefore provides stronger evidence that *G. tricostalis* is a single species that has a highly restricted host range to fireweed and its close *Senecio* relatives in South Africa, which has positive implications for this insect as a candidate biocontrol agent for fireweed in Australia.

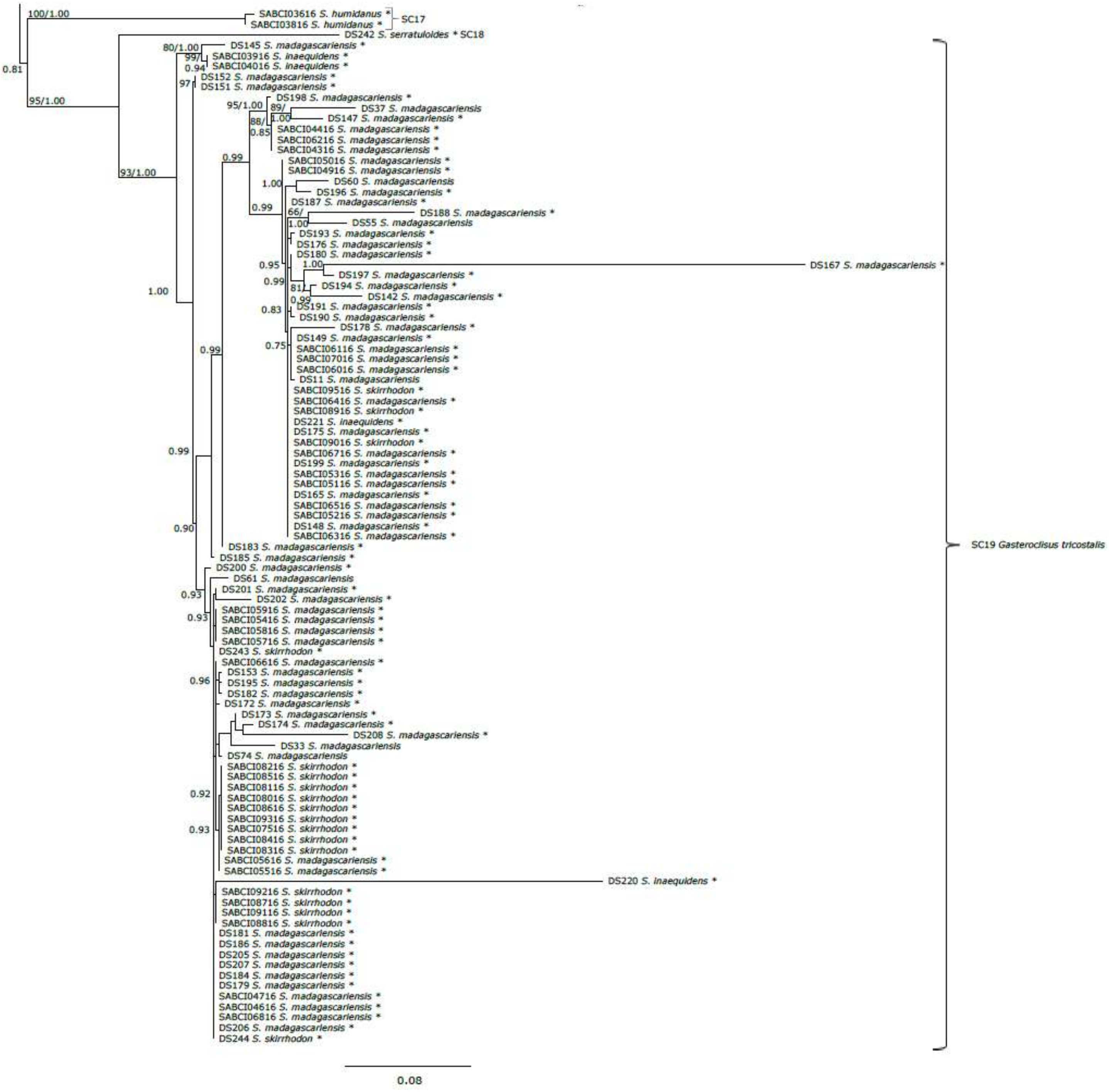


Figure 24. Mid-point rooted maximum likelihood COI phylogeny for stem boring Curculionidae associated with *Senecio madagascariensis* and non-target native *Senecio* species (* denotes larval specimens) across South Africa. Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. *Senecio* species on which insect specimens were sampled are labelled at the end of the branches. Different species clusters (SCs) indicate different species. Note that the phylogeny has been truncated to only show the cluster of specimens identified as *G. tricostalis* (all other weevil taxa excluded), due to size limitation when reproducing the phylogeny in this report.

Table 9. Non-target *Senecio* species surveyed for stem-boring larvae at sites around KwaZulu-Natal, South Africa. Voucher specimens collected at each location are lodged in the John Bews Herbarium (NU) at the University of KwaZulu-Natal.

Species	Date	Site	GPS (S; E)	Habitat
Senecio adnatus DC.	25/11/2014	Sevontein Prison	30.2648° 30.5951°	Grassland
	09/12/2014	Correctional Services Prison Sevontein	29.7612° 30.1374°	Grassland
	22/12/2014	Mount Gilboa	29.2856° 30.2926°	Grassland
	05/03/2018	Mount Gilboa	29.2857° 30.2927°	Grassland
	04/04/2018	Sevontein Prison	29.7642° 30.1513°	Dry savannah
Senecio affinis DC.	20/01/2014	Bellevue	29.6356° 30.4337°	Grassland
	18/02/2014	Emanzini	29.4684° 30.3709°	Savannah
	08/01/2015	Ukulinga	29.6668° 30.4031°	Grassland
	27/03/2018	Cumberland Nature Reserve	29.5186° 30.5145°	Grassland
Senecio bupleuroides DC.	08/10/2014	Hilton Road	29.5066° 30.2889°	Roadside
	12/11/2014	Ingonmankulu	29.7692° 30.4711°	Grassland
	26/11/2014	Bishopstowe	29.5762° 30.4306°	Roadside
	19/03/2018	Hilton College	29.4674° 30.2883°	Savannah
Senecio coronatus (Thunb.) Harv.	12/11/2014	Ukulinga	29.6668° 30.4031°	Grassland
	12/12/2014	Near Emanzini	29.4847° 30.3645°	Savannah
	13/12/2017	Ashburton Model Airplane Club	29.6792° 30.5084°	Dry savannah

	04/03/2018	Umgeni Valley	29.4760° 30.2449°	Grassland
Senecio glaberrimus DC.	08/10/2014	Hilton College	29.5135° 30.3006°	Grassland
	02/12/2014	Karkloof Conservation Centre	29.3461° 30.2921°	Grassland
	09/12/2014	Sevontein Prison	29.7612° 30.1374°	Grassland
	05/03/2018	Mount Gilboa	29.2857° 30.2927°	Grassland
	19/03/2018	Hilton College	29.4674° 30.2883°	Savannah
Senecio harveianus MacOwan	24/05/2014	Garden Castle	29.7474° 29.2080°	Grassland
	08/06/2014	Kamberg	29.4258° 29.7559°	Roadside
	01/03/2015	Sani Pass	29.5896° 29.2948°	Roadside
Senecio humidanus Cuatrec.	04/01/2015	Garden Castle	29.7554° 29.2292°	Grassland
	05/01/2015	Sani Pass (Bottom)	29.6462° 29.4296°	Grassland
	13/01/2014	Misty Valley Farm	29.4215° 30.1818°	Grassland
Senecio inaequidens DC.	08/06/2014	Lotheni	29.4868° 29.7019°	Roadside
	08/06/2014	Near Himeville	29.5297° 29.6224°	Roadside
	22/11/2014	Monk’s Cowl	29.0494° 29.4042°	Roadside
	20/02/2018	Drakensberg 1	29.3850° 29.9022°	Roadside
	20/02/2018	Drakensberg 2	29.4525° 29.7652°	Roadside
	20/02/2018	Drakensberg 3	29.5230° 29.6461°	Roadside

Senecio inornatus DC.	20/01/2014	Bellevue	29.6356° 30.4337°	Grassland
	20/03/2014	Hilton	29.4991° 30.3206°	Grassland
	12/02/2015	Umngeni Valley	29.4759° 30.2467°	Grassland
	20/02/2018	Drakensberg 4	29.4030° 29.8644°	Roadside
	20/02/2018	Drakensberg 5	29.6463° 29.5461°	Roadside
Senecio madagascariensis Poir.	20/01/2014	Bellevue	29.6356° 30.4337°	Grassland
	20/03/2014	Hilton	29.4991° 30.3206°	Grassland
	25/11/2014	Outside Vernon Crookes Reserve	30.2997° 30.6245°	Roadside
Senecio oxyriifolius DC.	14/10/2014	Hilton College	29.5135° 30.3006°	Grassland
	02/12/2014	Karkloof Conservation Centre	29.3461° 30.2921°	Grassland
	22/12/2014	Mount Gilboa	29.2856° 30.2926°	Grassland
	05/03/2018	Mount Gilboa	29.2857° 30.2927°	Grassland
Senecio panduriformis Hilliard.	04/04/2018	Sevontein Prison	29.7688° 30.1616°	Grassland
Senecio polyanthemoides Sch.Bip	20/02/2014	Bellevue	29.6356° 30.4337°	Savannah
	21/01/2014	Hillcrest	29.7585° 30.7828°	Roadside
	20/03/2014	Hilton	29.4991° 30.3206°	Forest edge
	16/12/2017	Ground Cover	29.3886° 30.1750°	Grassland
	04/03/2018	Umgeni Valley	29.4760° 30.2449°	Grassland

	19/03/2018	Hilton College	29.5082° 30.3089°	Grassland
	19/04/2018	Sevontein Prison	29.7643° 30.1457°	Grassland
Senecio retrorsus DC.	20/01/2014	Bellevue	29.6356° 30.4337°	Savannah
	25/01/2014	University of KZN	29.6261° 30.3966°	Grassland
	13/12/2017	Ashburton Model Airplane Club	29.6792° 30.5084°	Dry savannah
Senecio serratuloides DC.	19/03/2018	Hilton College	29.5082° 30.3089°	Wetland
	04/04/2018	Sevontein Prison	29.7564° 30.1562°	Wetland
Senecio skirrhodon DC.	13/04/2014	Mtwalume	30.4995° 30.6297°	Edge of sand dune
	25/11/2014	Park Rynie	30.3262° 30.7377°	Edge of sand dune
	01/02/2015	Hibberdene	30.5747° 30.5753°	Edge of sand dune
	06/02/2018	Mtwalume	30.4912° 30.6334°	Edge of sand dune
Senecio scitus Hutch. & Burtt Davy	29/11/2014	Sani Pass Hotel	29.6675° 29.4582°	Grassland
	09/12/2014	Sevontein Prison	29.7612° 30.1374°	Grassland
	04/01/2015	Garden Castle	29.7554° 29.2292°	Grassland
Senecio sp. nr. *adnatus* DC. 1	09/12/2014	Sevontein Prison	29.7612° 30.1374°	Grassland
	05/01/2015	Garden Castle	29.7554° 29.2292°	Grassland
	05/01/2015	Sani Pass	29.6462° 29.4296°	Grassland
	16/12/2017	Ground Cover	29.3917° 30.1777°	Dry grassland

	20/02/2018	Drakensberg 3	29.4525° 29.7652°	Roadside
Senecio sp. nr. *affinis* DC.	04/03/2018	Umgeni Valley	29.4794° 30.2476°	Grassland
Senecio sp. nr. *conrathii* N.E. Br.	25/11/2014	Vernon Crookes Nature Reserve	30.2648° 30.5951°	Savannah
	16/12/2017	Ground Cover	29.3934° 30.1742°	Dry grassland
	19/03/2018	Hilton College	29.5032° 30.3102°	Roadside/ grassland
Senecio sp. nr. *retrorsus* DC.	17/04/2014	Hilton	29.4991° 30.3206°	Grassland
Senecio sp. nr *serratuloides* DC.	19/03/2018	Hilton College	29.5082° 30.3089°	Wetland
	04/04/2018	Sevontein Prison	29.7462° 30.1546°	Edge of river/ grassland

5 Design and implementation of a host-utilisation experiment at the University of KwaZulu-Natal to determine the impacts of the weevil *Gasteroclisus tricostalis* on the growth of its preferred host plant, fireweed.

5.1 Context

Despite significant investment over several years into exploratory surveys of fireweed's natural enemies in its native South African range, to date there has been no empirical research (e.g., controlled experimentation) undertaken to test the hypothesis that the utilisation of fireweed as a food resource by the weevil *G. tricostalis* reduces the growth and reproductive output of the host plant. Even if the weevil is proved to be sufficiently host-specific to fireweed and, therefore, safe for release into Australia, its suitability as a biocontrol agent for fireweed must also consider its potential negative impacts on the target weed in the field.

A controlled larval impact experiment was undertaken at the UKZN laboratories in South Africa, with the aim of quantifying the magnitude of impact of larval density and feeding on the growth and reproductive output of fireweed. We also sought to evaluate the effects of oviposition density on larval development.

5.2 Methods

Experimental design

The experiment was conducted between 14th February and 18th May 2022 (late summer-autumn), coinciding with peak density of weevil larvae in the field (Singh et al., 2022). The experiment consisted of 10 trials, with each trial comprising of 4-7 fireweed test plants (with different densities of weevil larvae; described below) and a single fireweed control plant (with no larvae). Altogether, the experiment comprised 10 larval-free control plants and 50 larval-infested test plants.

The fireweed plants were grown in 17.5 cm pots and maintained in a shade house in the UKZN Botanical Garden until they had reached a reproductive maturity (i.e., > 35 cm in height and > 3 mm in stem diameter). The plants were then transferred to the UKZN insectary for exposure to the weevils. Prior to their exposure to adult weevils, we recorded maximum plant height, stem diameter, and the number of lateral shoots arising from the central stem, leaves, and capitulae (flower heads) for each individual plant. All plants included in each trial were similar in size and reproductive status prior to exposure to the weevils.

A pair of reproductively active weevils was placed on each test plant for either two, five, or seven days to acquire a range of egg-laying densities. A plastic sleeve was placed over each plant to confine each weevil pair. Following exposure to the adult weevils, the number of oviposition scars was recorded, and the plants were returned to the outdoor shade house to facilitate plant growth and larval development under more natural conditions.

After eight weeks, all plants were returned to the insectary and the plant size and reproduction variables were measured again. Floral material (i.e., inflorescences, capitulae) was removed from each plant and placed into brown paper bags. Each plant was cut at the base of the stem and the roots were washed to remove the soil and placed into brown paper bags. The stems were then dissected, and the late-instar larvae were recorded and preserved in 1.5 ml Eppendorf tubes with 100% ethanol. The dissected stems and leaf material were also placed into brown paper bags. The plant tissues (i.e., roots, floral material, and stems/leaves) were oven-dried at 60 °C for 24 hours, then weighed to determine the below-ground, above-ground, and floral biomass (g).

Statistical analysis

To analyse the effects of different larval densities on fireweed growth and reproduction, we first calculated the magnitude of change in each variable over time for each individual plant (i.e., value after 8 weeks minus baseline value prior to exposure to each weevil pair). Linear regression analysis was used to assess the relationship between the different plant growth and reproduction variables and larval density. The relationship between the numbers of eggs deposited per plant and percentage larval survival was determined by Spearman's rank-order correlation, since the data did not meet the assumptions of normality. Statistical analyses were conducted using IBM SPSS version 27.0.

5.3 Results

Weevil fecundity and larval loads

Daily fecundity was similar between the eight female weevils and ranged from 1-5 eggs per day of exposure to the 50 experimental plants, with a mean (± SE) of 2.0 ± 0.1 eggs laid per day. Over the varying periods of exposure (2-7 days), the number of eggs recovered from the test plants ranged from 1-19 per plant. However, there was considerable variation in oviposition viability per female, with larval recoveries at the end of each trial ranging from 5.6 % to 73.7 % of the eggs deposited. Consequently, there were significant differences between female weevils in the proportion of eggs that produced late-instar larvae ($\chi^2 = 22.403$; df = 7; P = 0.002). Although proportionally fewer larvae were produced after the 7-day adult exposure period (mean ± SE = 15.18 ± 10.30%) relative to the 5-day (41.77 ± 5.77%) and 2-day (46.11 ± 7.07%) exposure periods, the differences were not significant ($\chi^2 = 5.224$; df = 2; P = 0.073), with no significant interaction between female weevil and exposure time ($\chi 2 = 8.460$; df = 10; P = 0.584).

There was a significant negative relationship ($P < 0.001$) between the percentage of eggs that resulted in larval recoveries and the number of eggs deposited per plant (**Figure 25**). We speculate that this negative association reflects increased larval competition (aggression) with increasing oviposition density.

Plant responses

At the termination of the trials, 24 % of the weevil-exposed plants displayed clear signs of diminished health, with one dead plant and 11 plants displaying severe stem breakage. These plants typically contained three or more larvae. There were significant effects of increasing larval density on fireweed shoot and capitulum production, but not on plant height or leaf production (**Table 10, Figure 26**). From the regression equations, it was estimated that six larvae in each stem could prevent the production of new capitula by a plant, while 12 larvae could prevent the production of new shoots. There were no significant associations between larval loads and root or above-ground plant biomass (**Table 10; Figure 27**).

Despite the significant negative relationship between shoot production and larval density, there was no significant effect of larval density on shoot mortality ($\chi^2 = 3.996$; df = 3; P = 0.677) (**Figure 28a**). In contrast, there was a significant increase in capitulum mortality with increasing larval density ($\chi^2 = 16.175$; df = 6; P = 0.013) (**Figure 28b**).

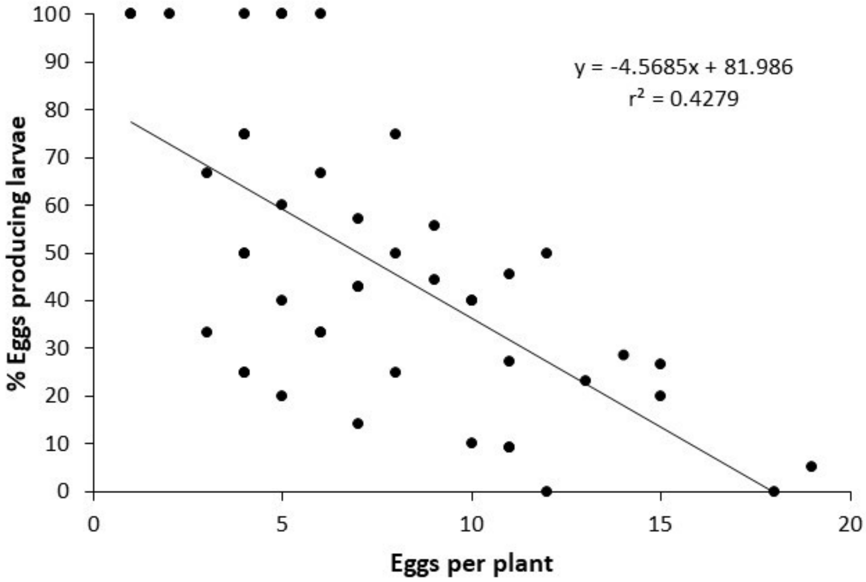


Figure 25. Percentage of eggs that produced late-instar larvae in relation to the number of eggs deposited by individual females of *Gasteroclisus tricostalis* on fireweed (*Senecio madagascariensis*) plants.

Table 10. Response of fireweed (*Senecio madagascariensis*) growth and reproductive variables (y) to stem boring by increasing numbers of *Gasteroclisus tricostalis* larvae (x).

Response variable	**Regression equation**	**r value**	**R^2 value**	***P* value**	**Larvae***
Plant height	y = -0.441x + 4.894	0.152	0.023	0.246	-
Stem diameter	y = -0.002x + 0.113	0.033	0.001	0.802	-
Shoot production	y = -2.237 + 25.549	0.266	0.071	0.040	12
Leaf production	y = -6.039x + 333.732	0.068	0.005	0.606	-
Floral production	y = -6.902x + 38.978	0.352	0.124	0.006	6
Above-ground biomass	y = -0.046x + 5.223	0.062	0.004	0.636	-
Below-ground biomass	y = -0.035x + 1.111	0.151	0.023	0.250	-

* Estimated number needed to curtail increases in the response variable; excluded for non-significant equations (P > 0.05).

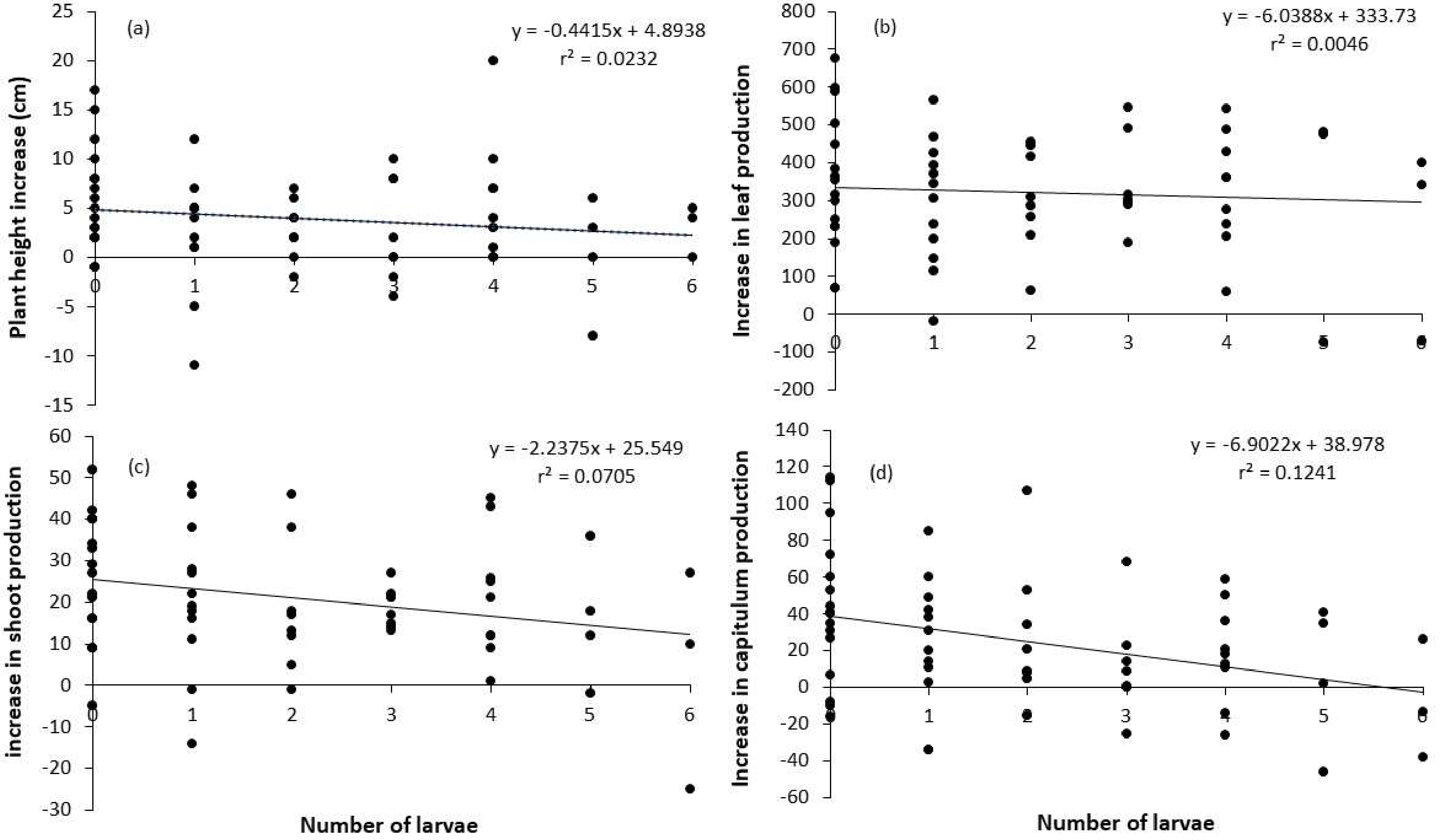


Figure 26. Increase in (a) plant height, (b) leaf production, (c) shoot production and (d) capitulum production by fireweed (*Senecio madagascariensis*) plants in relation to increasing numbers of *Gasteroclisus tricostalis* larvae.

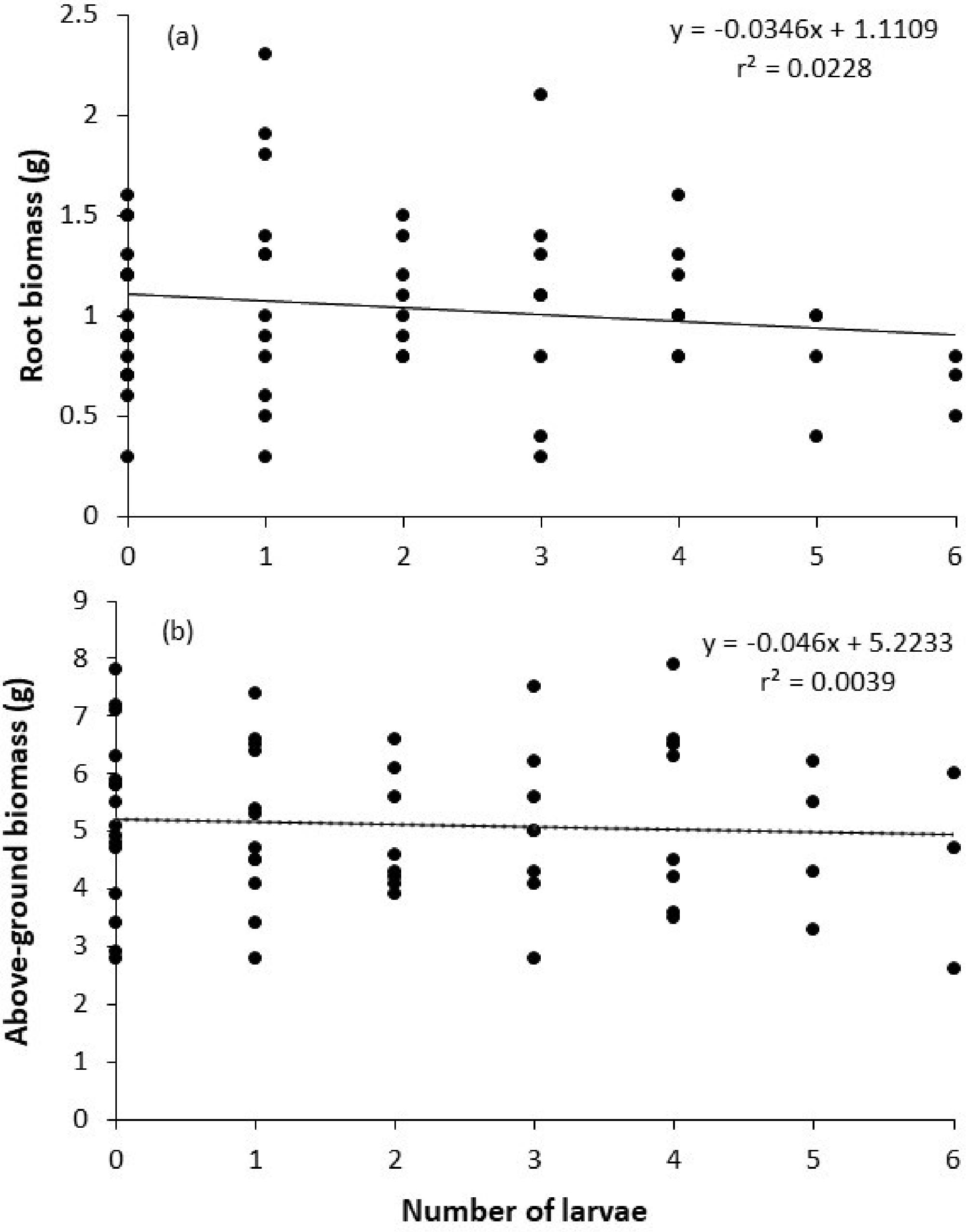


Figure 27. Biomass of the (a) roots and (b) above-ground material of fireweed (*Senecio madagascariensis*) plants in relation to increasing numbers of *Gasteroclisus tricostalis* larvae.

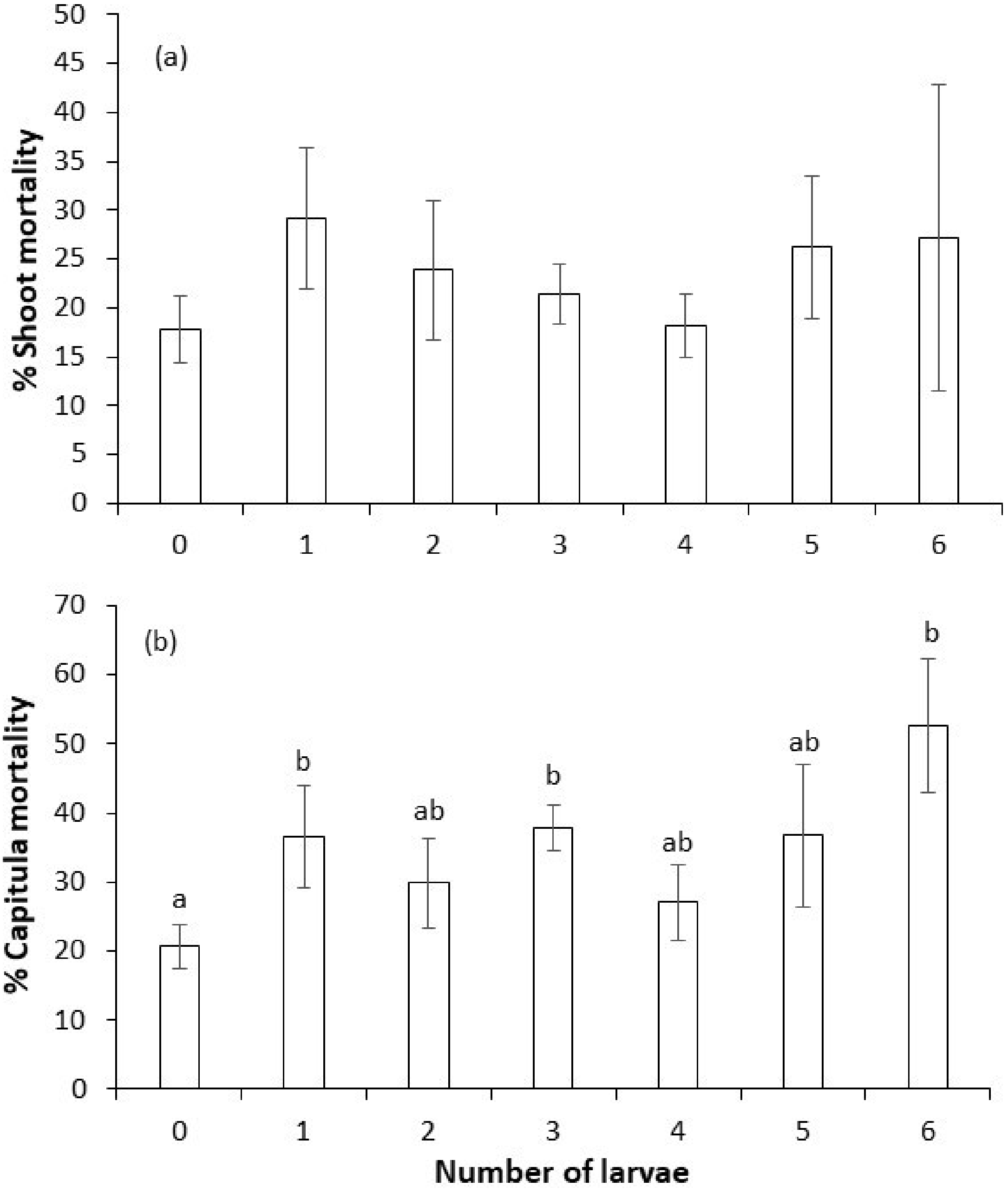


Figure 28. Percentage mortality suffered by the (a) shoots and (b) capitula of fireweed (*Senecio madagascariensis*) plants in relation to increasing number of *Gasteroclisus tricostalis* larvae. Different letters above the bars indicate significant differences (*P* < 0.05).

Stage 2 Evaluation of the lifecycle and development of a culturing methodology for the stem-boring moth *Metamesia elegans* in South Africa

1 Revising the host range of *Metamesia elegans* using phylogenetic analysis

1.1 Context

In this current project, we revised the host range associations of stem-boring moths within the *Senecio* genus across South Africa, with a focus on the tortricid moth *Metamesia elegans* (Walsingham) (Tortricidae, Lepidoptera). Our aim was to unite all existing data (collected previously between 2014 and 2018) into a single overarching phylogenetic analysis to ascertain whether *M. elegans* has a restricted native host range to fireweed and, if so, represents a suitable candidate biocontrol agent for the weed in Australia.

1.2 Methods

Twenty-two native South African *Senecio* species were surveyed between 2014 and 2015 (Egli *et al*. 2020), and then again between 2017 and 2018 (Singh *et al*. 2022) at several sites across the KwaZulu-Natal midlands, South Africa (**Table 11**). Between 5 to 10 whole plants were collected at each site to assess the host range of the larvae within the stems. An additional two plants of each species were collected at each site for lodgement in the John Bews Herbarium and purpose of identification using the key in *Compositae in Natal* (Hilliard 1977). Adult moths detected on each plant were also sampled for comparison with endophagous larvae.

Plants were uprooted and returned to the laboratory where they were frozen for later analysis under a dissecting microscope. All stem-boring lepidopteran larvae found within the stems were stored in 1.5 ml Eppendorf tubes with 100 % alcohol. Larval DNA was then extracted, and the cytochrome c oxidase subunit 1 mitochondrial (COI) gene sequenced at the Canadian Centre for DNA Barcoding. Phylogenetic analysis of the sequences then assigned these specimens to Species Clusters (tentative species or taxa) to determine host plant use by the lepidopteran species.

1.3 Results

A total of 73 lepidopteran COI barcode sequences were obtained from only four *Senecio* species: *S. madagascariensis* (57 sequences), *S. harveianus* (8), *S. inaequidens* (4) and *S. skirrhodon* (4). Of these, 12 were from adult lepidopterans and 61 were from larvae. The phylogenetic analysis revealed eight distinct species clusters (**Figure 29**). The largest of these, SC8, comprised 53 sequences (73%) belonging to *Metamesia elegans.* These individuals were collected on *S. madagascariensis* (48 specimens), *S. harveianus* (1), and *S, inaequidens* (4). All of these *Senecio* species are members of the *Senecio madagascariensis* species complex, which are more closely related to each other than any Australian native *Senecio* species. This suggests that *M. elegans* has host range that is highly restricted to fireweed and may represent a suitable secondary candidate biocontrol agent in addition to the top-priority candidate agent *G. tricostalis*.

Species cluster 5 comprised of nine sequences belonging to the pterophorid moth *Platyptilia* sp., which is both a capitulum borer and stem borer (**Figure 29**). *Platyptilia* sp. was mostly collected from fireweed (8 sequences), although one individual was collected on *S. harveianus*. *Platyptilia* sp. was collected more frequently in the capitulae and is discussed in more detail under Stage 3 below. Of the remaining six species clusters, three were made up of a single specimen, one cluster comprised two specimens and two clusters comprised three specimens (all specimens unknown, and possibly representing undescribed insect taxa).

Table 11. Non-target *Senecio* species surveyed for stem-boring larvae at sites around KwaZulu-Natal, South Africa between 2014-18. Voucher specimens collected at each location are lodged in the John Bews Herbarium (NU) at the University of KwaZulu-Natal.

Species	Date	Site	GPS (S; E)	Habitat
Senecio adnatus DC.	25/11/2014	Vernon Crookes Reserve	30.264866° 30.595133°	Grassland
	09/12/2014	Sevontein Prison	29.761278° 30.137408°	Grassland
	22/12/2014	Mount Gilboa	29.285622° 30.292696°	Grassland
	05/03/2018	Mount Gilboa	29.28572° 30.292756°	Grassland
	04/04/2018	Sevontein Prison	29.764279° 30.151354°	Dry savannah
Senecio affinis DC.	20/01/2014	Bellevue	29.635608° 30.43374°	Grassland
	18/02/2014	Emanzini	29.468478° 30.370984°	Savannah
	08/01/2015	Ukulinga	29.66684° 30.403181°	Grassland
	27/03/2018	Cumberland Nature Reserve	29.51869° 30.51451°	Grassland
Senecio bupleuroides DC.	08/10/2014	Hilton Road	29.506672° 30.288927°	Roadside
	12/11/2014	Ingonmankulu	29.769228° 30.471163°	Grassland
	26/11/2014	Bishopstowe	29.576262° 30.430695°	Roadside
	19/03/2018	Hilton College	29.467476° 30.288355°	Savannah
Senecio coronatus (Thunb.) Harv.	12/11/2014	Ukulinga	29.66684° 30.403181°	Grassland
	12/12/2014	Near Emanzini	29.484783° 30.364517°	Savannah
	13/12/2017	Ashburton Model Airplane Club	29.679253° 30.508434°	Dry savannah
	04/03/2018	Umgeni Valley	29.476005° 30.244931°	Grassland

Senecio glaberrimus DC.	08/10/2014	Hilton College	29.513508° 30.300622°	Grassland
	02/12/2014	Karkloof Conservation Centre	29.346162° 30.292155°	Grassland
	09/12/2014	Sevontein Prison	29.761278° 30.137408°	Grassland
	05/03/2018	Mount Gilboa	29.28572° 30.292756°	Grassland
	19/03/2018	Hilton College	29.467476° 30.288355°	Savannah
Senecio harveianus MacOwan	24/05/2014	Garden Castle	29.74749° 29.20803°	Grassland
	08/06/2014	Kamberg	29.42586° 29.75595°	Roadside
	01/03/2015	Sani Pass	29.589648° 29.294886°	Roadside
Senecio humidanus C. Jeffrey	04/01/2015	Garden Castle	29.755434° 29.229294°	Grassland
	05/01/2015	Sani Pass (Bottom)	29.646286° 29.429642°	Grassland
	13/01/2014	Misty Valley Farm	29.421546° 30.181881°	Grassland
Senecio inaequidens DC.	08/06/2014	Lotheni	29.48687° 29.70194°	Roadside
	08/06/2014	Near Himeville	29.52972° 29.62247°	Roadside
	22/11/2014	Monk’s Cowl	29.049478° 29.404205°	Roadside
	20/02/2018	Drakensberg 1	29.385° 29.902222°	Roadside
	20/02/2018	Drakensberg 2	29.4525° 29.765278°	Roadside
	20/02/2018	Drakensberg 3	29.523056° 29.646111°	Roadside
Senecio inornatus DC.	20/01/2014	Bellevue	29.635608° 30.43374°	Grassland
	20/03/2014	Hilton	29.499149° 30.320662°	Grassland
	12/02/2015	Umngeni Valley	29.475918° 30.246727°	Grassland
	20/02/2018	Drakensberg 4	29.403056° 29.864444°	Roadside
	20/02/2018	Drakensberg 5	29.646309° 29.546111°	Roadside
Senecio madagascariensis Poir.	20/01/2014	Bellevue	29.635608° 30.43374°	Grassland
	20/03/2014	Hilton	29.499149°	Grassland

			30.320662°	
	25/11/2014	Outside Vernon Crookes Reserve	30.299717° 30.624505°	Roadside
Senecio oxyriifolius DC.	14/10/2014	Hilton College	29.513508° 30.300622°	Grassland
	02/12/2014	Karkloof Conservation Centre	29.346162° 30.292155°	Grassland
	22/12/2014	Mount Gilboa	29.285622° 30.292696°	Grassland
	05/03/2018	Mount Gilboa	29.28572° 30.292756°	Grassland
Senecio panduriformis Hilliard.	04/04/2018	Sevontein Prison	29.768899° 30.161616°	Grassland
Senecio polyanthemoides Sch.Bip	20/02/2014	Bellevue	29.635608° 30.43374°	Savannah
	21/01/2014	Hillcrest	29.75854° 30.78285°	Roadside
	20/03/2014	Hilton	29.499149° 30.320662°	Forest edge
	16/12/2017	Ground Cover	29.38863° 30.175074°	Grassland
	04/03/2018	Umgeni Valley	29.476005° 30.244931°	Grassland
	19/03/2018	Hilton College	29.508225° 30.308938°	Grassland
	19/04/2018	Sevontein Prison	29.764325° 30.14576°	Grassland
Senecio retrorsus DC.	20/01/2014	Bellevue	29.635608° 30.43374°	Savannah
	25/01/2014	University of KZN	29.626191° 30.396641°	Grassland
	13/12/2017	Ashburton Model Airplane Club	29.679253° 30.508434°	Dry savannah
Senecio scitus Hutch. & Burtt Davy	29/11/2014	Sani Pass Hotel	29.667503° 29.458283°	Grassland
	09/12/2014	Sevontein Prison	29.761278° 30.137408°	Grassland
	04/01/2015	Garden Castle	29.755434° 29.229294°	Grassland
Senecio serratuloides DC.	19/03/2018	Hilton College	29.508225° 30.308938°	Wetland
	04/04/2018	Sevontein Prison	29.756406°	Wetland

			30.156252°	
Senecio skirrhodon DC.	13/04/2014	Mtwalume	30.499567° 30.629719°	Edge of sand dune
	25/11/2014	Park Rynie	30.326279° 30.737762°	Edge of sand dune
	01/02/2015	Hibberdene	30.574742° 30.575346°	Edge of sand dune
	06/02/2018	Mtwalume	30.491218° 30.633444°	Edge of sand dune
Senecio sp. nr. *adnatus* DC.	16/12/2017	Ground Cover	29.391786° 30.177781°	Dry grassland
	20/02/2018	Drakensberg 3	29.4525° 29.765278°	Roadside
Senecio sp. nr. *adnatus* DC. 1	09/12/2014	Sevontein Prison	29.761278° 30.137408°	Grassland
	05/01/2015	Garden Castle	29.755434° 29.229294°	Grassland
	05/01/2015	Sani Pass	29.646286° 29.429642°	Grassland
Senecio sp. nr. *affinis* DC.	04/03/2018	Umgeni Valley	29.479455° 30.247691°	Grassland
Senecio sp. nr. *conrathii* N.E. Br.	25/11/2014	Vernon Crookes Reserve	30.264866° 30.595133°	Savannah
	16/12/2017	Ground Cover	29.393459° 30.174269°	Dry grassland
	19/03/2018	Hilton College	29.503217° 30.310248°	Roadside/ grassland
Senecio sp. nr. *retrorsus* DC.	17/04/2014	Hilton	29.499149° 30.320662°	Grassland
Senecio sp. nr. *serratuloides* DC.	19/03/2018	Hilton College	29.508225° 30.308938°	Wetland
	04/04/2018	Sevontein Prison	29.746293° 30.154656°	Edge of river/ grassland

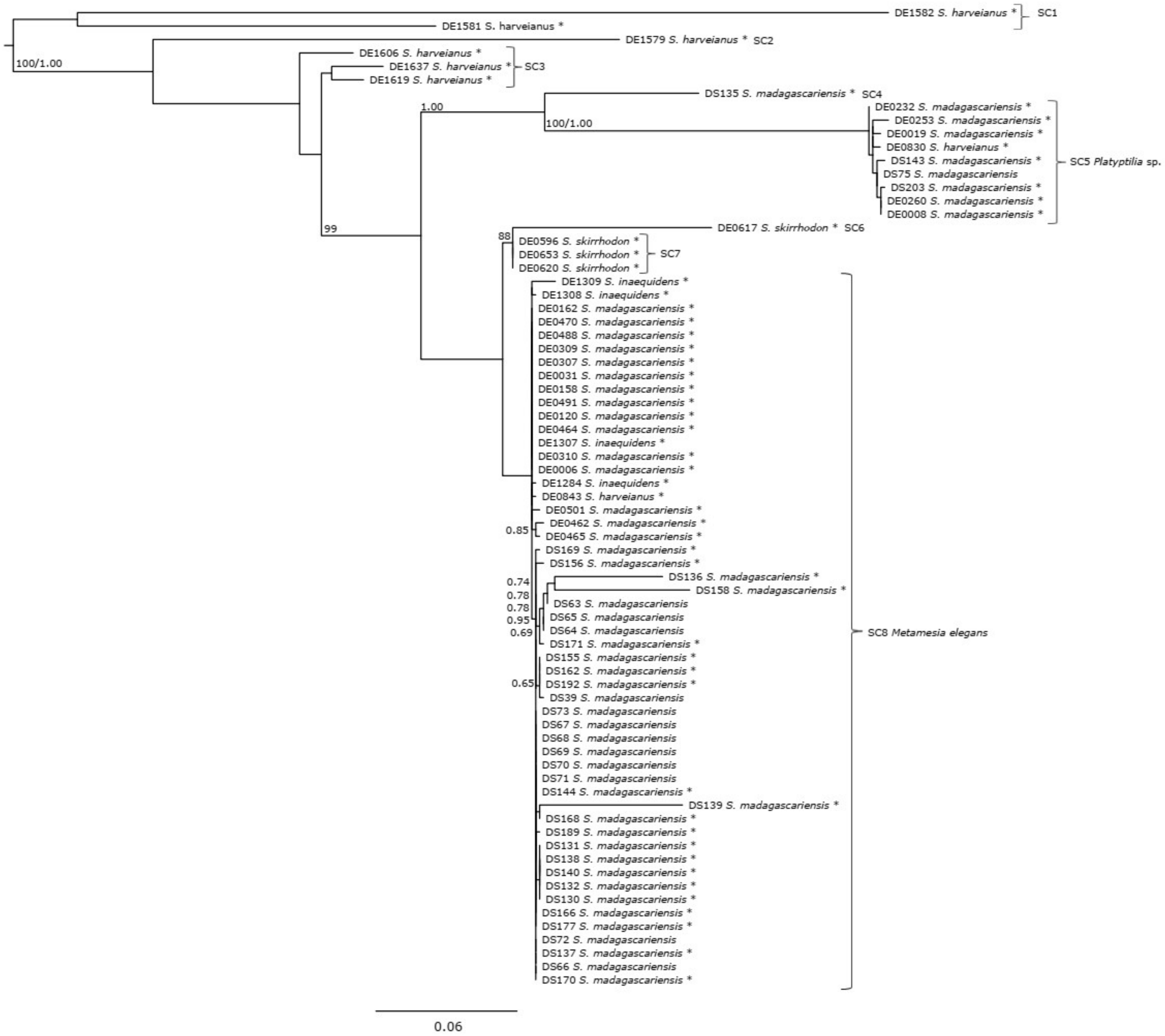


Figure 29. Mid-point rooted maximum likelihood COI phylogeny for stem-boring Lepidoptera associated with *Senecio madagascariensis* and non-target *Senecio* species (* denotes larval specimens). Values on branches indicate bootstrap values and posterior probability values. Only bootstrap values ≥ 65% and posterior probability ≥ 0.65 are shown. *Senecio* species are labelled at the end of the branches Different operational taxonomic units or species clusters (SCs) indicate different species.

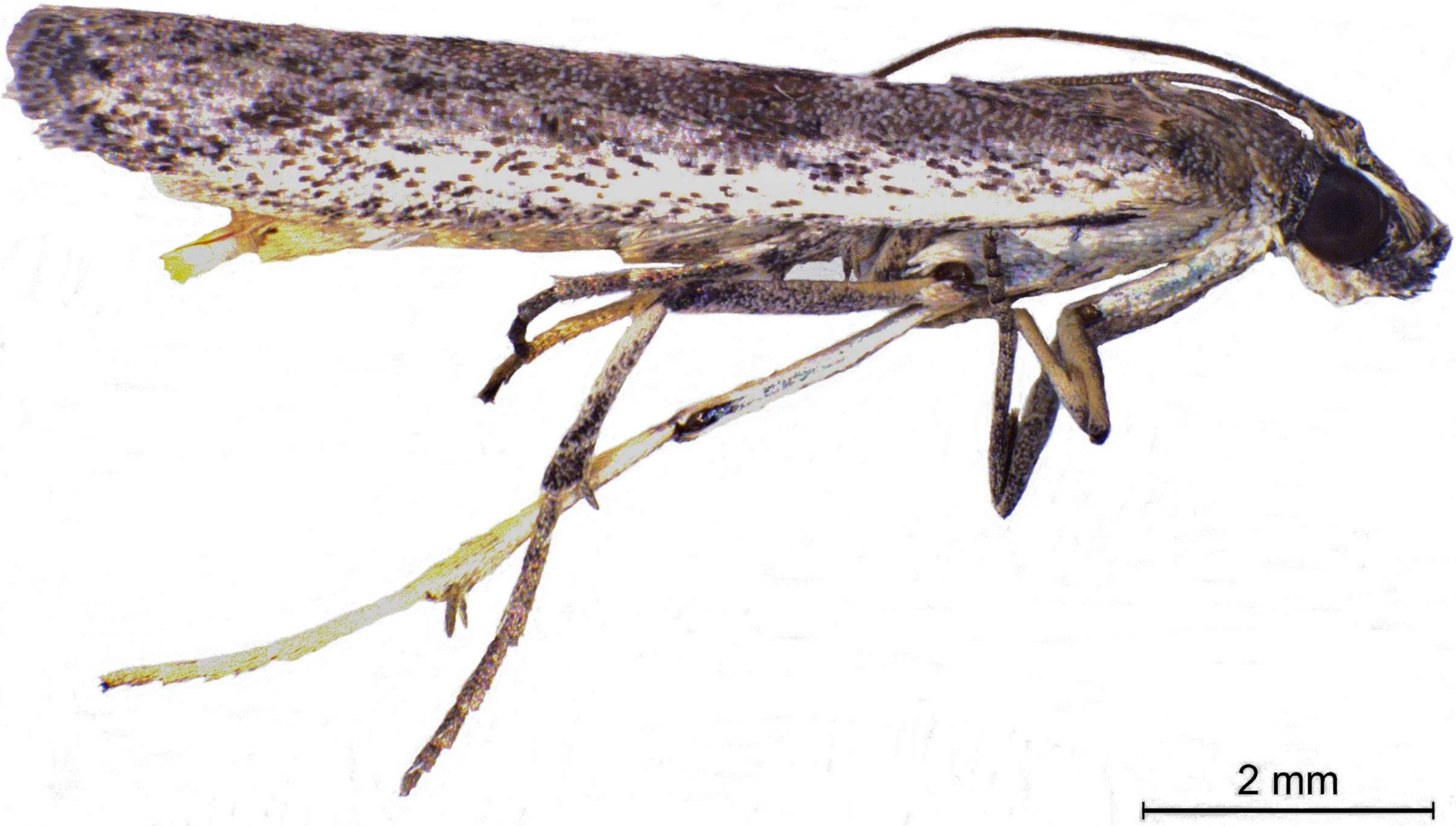
2 Developing a lab-reared culture of *Metamesia elegans*

In an attempt to establish an *M. elegans* culture at the UKZN laboratories in South Africa, 28 field trips were undertaken between June 2020 and December 2021 (same field excursions as for collection of *G. tricostalis*, see **Table 1**). On each occasion, between three to six fireweed plants were uprooted and placed in jars of water housed in BugDorm cages in the insectary to rear adult moths. In August 2021, a single adult *M. elegans* moth emerged from the field-collected plants in the laboratory.

As tortricid moths are typically nocturnal, we also attempted to sample adult moths from one site (Emanzini) between 9.30 pm and midnight by hanging a white sheet and a spotlight shone over a large patch of fireweed plants. These plants were also swept with a sweep net. However, no *M. elegans* adults were reared from these field-collected plants, nor were any adults collected during the night surveys.

The almost complete absence of adult *M. elegans* moths from the field or reared in the laboratory from the field-collected plants between June 2020 and December 2021 was puzzling. This may have arisen either because of a commensurate absence of larvae within the sampled fireweed populations or the presence of larvae that for some reason had failed to complete their development to adulthood. To address this question, a total of 20 fireweed plants were uprooted in February and March 2022 from Ashburton and Ukulinga, brought back to the laboratory and dissected. Only one *M. elegans* larva was detected on a plant collected from Ashburton, thus indicating that there was indeed low abundance of this moth species in the environment during the sampling period.

Stage 3 Refinement of the host ranges of capitulum-feeding insects in South Africa



1 Revised phylogenetic analysis to elucidate host ranges of capitulum-feeding insects (focussing on pyralid and pterophorid moths) across South Africa

1.1 Context

Previous research (see Egli & Olckers 2015, 2017, 2020; Egli et al 2020) identified the capitulum-feeding pyralid moth *Homoeosoma stenotea* (Lepidoptera: Pyralidae) and the capitulum- and stem-boring moth *Platyptilia* sp. (Lepidoptera: Pterophoridae) as potential candidate biocontrol agents for fireweed in Australia, yet further research was required to verify their restricted host ranges to fireweed across the native South African range.

The aim of this current project was to unite all existing DNA (COI barcode) sequence data collected between 2014 and 2020 into a single, unified phylogenetic analysis, to ascertain the host ranges of capitulum-feeding moths associated with fireweed in the native South African range.

Furthermore, we also sampled the capitulum-feeding Lepidoptera, Coleoptera and Diptera associated with *Senecio madagascariensis* each month to determine the seasonal variability in the relative abundance of key insect taxa. Such seasonal data can inform the development of culturing methods, and the timing and location of field collections.

1.2 Methods

Host range analysis

To determine the host plant affinities of these two target moth species, exploratory surveys of lepidopteran larvae associated with capitulae were undertaken across South Africa for 34 non-target *Senecio* species (sample details presented in **Table 12**). At each site, between five to 13 plants were collected depending on plant availability. Capitulae were removed from plants, counted, and frozen for later inspection that involved dissection under a microscope. Lepidopteran larvae were counted and stored in 100 % ethanol for DNA sequencing. At each site, two additional plants were collected – one was pressed and lodged in the John Bews Herbarium (NU), and one was used to identify the plant species using the key presented in *Compositae in Natal* (Hilliard 1977).

Seasonal abundance surveys

Four populations of fireweed (**Table 13**) were surveyed monthly for one year (April 2018-March 2019) to determine the relative seasonal abundance of capitulum-feeding insects. The two higher altitude sites (Cedara and Groundcover) were cooler and wetter, and the two lower altitude sites (Ukulinga and Raptor Centre) were drier and warmer. At each site, five mature fireweed plants were collected every month; thus, a total of 240 plants (5 plants x 4 sites x 12 months) were sampled during the study period. The floral material was removed from each plant and frozen for later inspection. The capitulae were counted and then dissected under a microscope. All insect larvae were removed and stored in 100 % alcohol for DNA sequencing.

In addition, in every month at each site, three mature *Senecio madagascariensis* plants were collected and returned to the lab. The capitulae were removed and placed in ventilated plastic containers to rear any constituent insect larvae to adulthood. Adults were identified taxonomically as much as possible and matched to the larvae by comparing DNA sequences (described below). Voucher specimens of the Lepidoptera are lodged with the Durban Natural Science Museum.

DNA sequencing

Lepidopteran larvae collected from both the host range and seasonal surveys were added to a 96 well microplate and sent to the Canadian Centre for DNA Barcoding for Sanger sequencing of the COI gene (accession data provided in **Table 14**). A revised phylogenetic analysis was undertaken for all larval sequences obtained from both the host range and seasonal surveys. Within the phylogeny, specimens were assigned to species clusters (SCs), which are equivalent to species when taxonomic information is lacking.

1.3 Results

Host range analysis

A total of 62 lepidopteran specimens were sequenced from eight host plant species (**Tables 14** and **15**). The phylogenetic analysis revealed 14 species clusters, with most of the lepidopterans belonging to SC 9 (*H. stenotea*; 27 sequences) and SC 14 (*Platyptilia* species; 18 sequences) (**Figure 30**). *Homeosoma stenotea* was recorded from seven *Senecio* species: fireweed (13 individuals, 28 %), *S. bupleuroides* (1 individual), *S. coronatus* (3 individuals), *S. glabberimus* (2 individuals), *S. inaequidens* (6 individuals), *S. oxyriifolius* (1 individual), and *S. polyanthemoides* (1 individual). *Platyptilia* sp. was recorded on three *Senecio* species: fireweed (15 individuals, 83 %), *S. harveianus* (1 individual) and *S. polyodon* (2 individuals). An additional five unidentified lepidopteran species clusters were recorded on fireweed. These all were made up of low numbers of larvae, with three clusters comprising a single specimen, one cluster represented by two specimens and one cluster represented by three specimens.

Seasonal abundance surveys

The capitulum-feeders that were dissected from fireweed belonged to the orders Lepidoptera, Diptera and Coleoptera. The most common taxa belonged to Coleoptera, which made up 72.8 % of the larvae sampled (**Figure 31**). Lepidoptera made up 15.8 % and Diptera 11.4 %. The number of capitulae available was fairly constant at Ukulinga, but highly variable at the other three sites (**Figure 31**). Numbers of capitulae available varied from less than 10 to over 120 per plant.

Lepidopteran larvae were collected from several sites during 8 months of the year, though numbers were typically quite low (**Figure 31**). No lepidopteran larvae were recovered from capitulae during July, November, December, and January. The greatest numbers were recovered from February to May. Lepidopteran adults were reared throughout most of the year as well, with the highest number of adults of *H. stenotea* recorded between August and November, and none reared in February, March, and June (**Figure 32**). *Platyptilia* sp. was less abundant and only reared from capitulae collected at two of the four sites over six months of the year (**Figure 32)**.

1.4 Conclusions

Although *H. stenotea* was present throughout most of the year, and appears to be quite damaging to fireweed's capitulae, its field host range is broad, with populations detected on several South African *Senecio* species that are more phylogenetically distantly related to fireweed than many native Australian *Senecio* species. We therefore consider that *H. stenotea* is unlikely to be sufficiently host-specific to fireweed to justify importing it into quarantine in Australia for future host specificity experimentation with native Australian *Senecio* species. This is comparable to the situation with a capitulum-feeding pyralid moth from the genus *Phycitodes* (Pyralidae), which has a similar biology to *H. stenotea*; the *Phycitodes* species was imported into Australian quarantine from Madagascar but was able to develop on several non-target plant species, and was thus rejected as a candidate biocontrol agent for fireweed (McFadyen and Morin, 2012).

Platyptilia sp., which is similarly damaging to capitulae but also bores into fireweed's stems, was considerably less abundant in the field compared to *H. stenotea* but had a much more restricted host range to fireweed. Although it was recorded in the capitulae of two non-target species, one of these (*S. harveianus*) is very closely related to fireweed and belongs to the same species complex. The other species, *S. polyodon*, was a single population that supported only two moth specimens. This may indicate that *S. polyodon* is not a common or preferred host of *Platyptilia* sp.; thus, it may be worthwhile to perform some preliminary host-specificity testing in a laboratory environment in South Africa before rejecting *Platyptilia* sp. as a candidate agent for fireweed in Australia.

Table 12. Plant species sampled for capitulum-feeding Lepidoptera, their sampling dates and locations. Voucher specimens from each location are lodged in the University of KwaZulu-Natal John Bews Herbarium (NU).

Species	Date	Location	Collector's No.*	Coordinates	Habitat
Senecio adnatus DC.	25-11-2014	Vernon Crooks Nature Reserve	D Egli 100	30°26'48''S 30°59'51''E	Grassland
	22-12-2014	Mount Gilboa	D Egli 131	29°28'56''S 30°29'27''E	Rocky grassland
	05-03-2018	Mount Gilboa	D Egli 183	29°28'57''S 30°29'28''E	Rocky grassland
	12-12-2019	Karkloof	D Egli 226	29°26'31''S 30°34'53''E	Roadside
	28-01-2020	Groundcover	D Egli 232	29°39'14''S 30°17'76''E	Roadside
Senecio affinis DC.	20-01-2014	Bellvue	D Egli 55	29°63'56''S 30°43'47''E	Savanna
	08-01-2015	Ukulinga Research Farm	D Egli 151	29°66'68''S 30°40'32''E	Grassland
	08-01-2015	Emanzini Private Reserve	D Egli 158	29°46'85''S 30°37'10''E	Grassland
	27-03-2018	Cumberland Nature Reserve	D Egli 195	29°51'87''S 30°51'45''E	Grassland
Senecio bupleuroides DC.	20-01-2014	Bellvue	D Egli 52	29°63'56''S 30°43'37''E	Savanna
	12-11-2014	Camperdown, Ingomankulu	D Egli 94	29°76'92''S 30°47'12''E	Grassland
	26-11-2014	Pietermaritzburg, Bishopstowe	D Egli 104	29°57'63''S 30°43'07''E	Roadside
	29-11-2014	Sani Pass Hotel	D Egli 108	29°66'75''S 29°45'83''E	Grassland
Senecio brevidentatus M.D.Hend	05-01-2015	Himeville	D Egli 147	29°60'43''S 29°34'53''E	Roadside
Senecio conrathii N.E.Br	09-12-2014	Boston	D Egli 116	29°76'13''S	Grassland

				30°13'74''E	
	05-12-2019	Mooi River	D Egli 225	29°24'77''S 29°99'58''E	Grassland
Senecio coronatus (Thumb.) Harv.	12-11-2014	Ukulinga Research Farm	D Egli 91	29°66'68''S 30°40'32''E	Grassland
	12-12-2014	Emanzini Private Reserve	D Egli 128	29°48'48''S 30°36'45''E	Grassland
	13-12-2017	Ashburton	D Egli 167	29°67'93''S 30°50'84''E	Grassland
	06-03-2018	Howick, Umngeni Valley	D Egli 186	29°47'60''S 30°24'49''E	Grassland
	28-10-2019	Ashburton Raptor Centre	D Egli 222	29°67'54''S 30°51'35''E	Grassland
Senecio decurrens DC.	02-12-2014	Karkloof	D Egli 124	29°34'61''S 30°29'21''E	Grassland
Senecio glaberrimus DC.	22-11-2014	Giants Castle	D Egli 97	30°29'21''E 29°42'52''E	Roadside
	22-12-2014	Mount Gilboa	D Egli 130	29°28'56''S 30°29'27''E	Rocky grassland
	05-03-2018	Mount Gilboa	D Egli 184	29°28'57''S 30°29'28''E	Rocky grassland
	12-12-2019	Karkloof	D Egli 227	29°26'31''S 30°34'53''E	Roadside
	12-12-2019	Mount Gilboa	D Egli 229	29°26'38''S 30°30'04''E	Rocky grassland
Senecio heliopsis Hilliard & B.L. Burtt	22-12-2014	Mount Gilboa	D Egli 133	29°28'56''S 30°29'27''E	Rocky grassland
Senecio humidanus C. Jeffrey	05-01-2015	Himeville	D Egli 142	29°64'63''S 29°42'96''E	Roadside
	13-01-2015	Curry's Post	D Egli 153	29°42'15''S 30°18'19''E	Grassland
Senecio inaequidens DC.	22-11-2014	Giants Castle	D Egli 96	29°04'95''S 29°40'42''E	Roadside
	02-02-2018	Impendle	D Egli 181	29°52'31''S	Roadside

				29°64'61''E	
	02-02-2018	Impendle	D Egli 179	29°45'25''S 29°76'53''E	Roadside
	02-02-2018	Nottingham Road	D Egli 177	29°38'50''S 29°90'22''E	Roadside
Senecio inornatus DC.	18-02-2014	Emanzini Private Reserve	D Egli 67	29°46'85''S 30°37'10''E	Savanna
	09-12-2014	Boston	D Egli 118	29°76'13''S 30°13'74''E	Grassland
	22-12-2014	Mount Gilboa	D Egli 132	29°28'56''S 30°29'27''E	Rocky grassland
	08-02-2015	Emanzini Private Reserve	D Egli 157	29°46'85''S 30°37'10''E	Grassland
	20-02-2018	Fort Nottingham	D Egli 178	29°40'31''S 29°86'44''E	Roadside
	20-02-2018	Himeville	D Egli 182	29°64'63''S 29°54'61''E	Roadside
Senecio isatidioides Phill. & Sm.	12-12-2019	Karkloof	D Egli 228	29°25'79''S 30°33'93''E	Roadside
	12-12-2019	Mount Gilboa	D Egli 231	29°24'52''S 30°27'96''E	Rocky grassland
Senecio madagascariensis Poir.	21-01-2014	Hillcrest	D Egli 56	29°75'85''S 30°78'29''E	Farmland
	21-01-2014	Summerveld	D Egli 58	29°80'22''S 30°70'95''E	Roadside
	07-02-2014	Pietermaritzburg	D Egli 61	29°62'62''S 30°39'66''E	Grassland
	13-02-2014	Nottingham Road	D Egli 62	29°38'60''S 30°03'94''E	Grassland
	14-02-2014	Wedgewood Nougat	D Egli 64	29°55'02''S 30°25'26''E	Cattle farm
	16-02-2014	Emanzini Private Reserve	D Egli 66	29°46'85''S 30°37'10''E	Savanna
	15-04-2014	Wartburg	D Egli 77	29°47'83''S	Cattle farm

				30°41'76''E	
	26-04-2014	Southbroom	D Egli 78	30°91'96''S 30°32'45''E	Roadside
	24-09-2014	Hluhluwe	D Egli 85	28°02'24''S 32°27'42''E	Roadside
	13-01-2015	Curry's Post	D Egli 154	29°42'15''S 30°18'19''E	Grassland
	29-01-2020	Mooi River	D Egli 241	29°24'77''S 29°99'58''E	Grassland
Senecio oxyriifolius DC.	09-12-2014	Boston	D Egli 119	29°76'13''S 30°13'74''E	Rocky grassland
	22-12-2014	Mount Gilboa	D Egli 129	29°28'56''S 30°29'27''E	Rocky grassland
Senecio panduriformis Hilliard	04-04-2018	Elandskop, Sevontein Prison	D Egli 198	29°76'89''S 30°16'16''E	Grassland
Senecio polyanthemoides Sch. Bip.	21-01-2014	Hillcrest	D Egli 57	29°75'85''S 30°78'29''E	Farmland
	22-01-2014	Ashburton Raptor Centre	D Egli 59	29°67'54''S 30°51'35''E	Grassland
	27-01-2014	Port Edward, Izingolweni Rd	D Egli 80	31°05'20''S 30°19'77''E	Roadside
	14-02-2014	Hilton, Wedgewood	D Egli 65	29°55'02''S 30°25'26''E	Cattle farm
	27-04-2014	Palm Beach	D Egli 79	30°99'21''S 30°26'17''E	Roadside
	13-01-2015	Curry's Post	D Egli 155	29°42'15''S 30°18'19''E	Grassland
	16-01-2018	Curry's Post	D Egli 169	29°38'86''S 30°17'51''E	Grassland
	06--03-2018	Howick, Umngeni Valley	D Egli 187	29°47'60''S 30°24'49''E	Grassland
	13-03-2018	Hilton	D Egli 191	29°50'82''S 30°30'89''E	Grassland
	04-04-2018	Elandskop, Sevontein Prison	D Egli 199	29°76'43''S	Pine forest

				30°14'58''E	
	12-12-2019	Mount Gilboa	D Egli 230	29°27'04''S 30°28'93''E	Rocky grassland
Senecio polyodon DC.	02-12-2014	Karkloof	D Egli 113	29°34’61’’S 30°29’21’’E	Grassland
Senecio retrorsus DC.	20-01-2014	Bellvue	D Egli 54	29°63'56''S 30°43'37''E	Savanna
	12-02-2015	Howick, Umngeni Valley	D Egli 162	29°47'59''S 30°24'67''E	Grassland
	13-12-2017	Ashburton	D Egli 168	29°67'93''S 30°50'84''E	Grassland
Senecio rhomboideus Harv.	05-01-2015	Himeville	D Egli 144	29°60'43''S 29°34'53''E	Roadside
Senecio scitus Hutch. & Burtt Davy	29-11-2014	Sani Pass Hotel	D Egli 107	29°66'75''S 29°45'83''E	Grassland
	04-01-2015	Garden Castle Reserve	D Egli 136	29°75'54''S 29°22'93''E	Grassland
	05-01-2015	Himeville	D Egli 148	29°60'43''S 29°34'53''E	Roadside
Senecio scoparius Harv.	12-02-2015	Howick, Umngeni Valley	D Egli 165	29°47'59''S 30°24'67''E	Grassland
Senecio serratuloides DC.	13-03-2018	Hilton	D Egli 192	29°50'82''S 30°30'89''E	Marshy area
	04-04-2018	Elandskop, Sevontein Prison	D Egli 200	29°75'64''S 30°15'63''E	Marshy area
Senecio skirrhodon DC.	13-04-2014	Mthwalume	D Egli 172	30°49'96''S 30°62'97''E	Edge of beach sand
	01-02-2015	Hibberdine	D Egli 156	30°57'47''S 30°57'53''E	Roadside
	06-02-2018	Mthwalume	D Egli 172	30°49'12''S 30°63'34''E	Sand dunes
Senecio sp. nr. *adnatus* DC.	16-01-2018	Curry’s Post	D Egli 170	29°39'18''S 30°17'78''E	Roadside
	02-02-2018	Impendle	D Egli 180	29°45'25''S	Roadside

				29°76'53''E	
Senecio sp. nr. *adnatus 2*	04-01-2015	Garden Castle Reserve	D Egli 135	29°75'54''S 29°22'93''E	Grassland
	05-01-2015	Garden Castle Reserve	D Egli 138	29°75'54''S 29°22'93''E	Grassland
	05-01-2015	Himeville	D Egli 146	29°60'43''S 29°34'53''E	Roadside
Senecio sp. nr. *affinis* DC.	06-03-2018	Howick, Umngeni Valley	D Egli 188	29°47'95''S 30°24'77''E	Grassland
Senecio sp. nr. *conrathii* N.E.Br	16-01-2018	Curry's Post	D Egli 171	29°39'35''S 30°17'43''E	Grassland
	13-03-2018	Hilton	D Egli 193	29°50'32''S 30°31'03''E	Roadside
Senecio sp. nr. *hastatus* L.	29-11-2014	Sani Pass Hotel	D Egli 106	29°66'75''S 29°45'83''E	Grassland
Senecio sp nr *serratuloides* DC.	13-03-2018	Hilton	D Egli 194	29°50'82''S 30°30'89''E	Marshy area
	04-04-2018	Elandskop, Sevontein Prison	D Egli 201	29°74'63''S 30°15'47''E	Marshy area
Senecio striatifolius DC.	02-12-2014	Karkloof	D Egli 109	29°34'61''S 30°29'21''E	Grassland
Senecio urophyllus Conrath.	05-01-2015	Himeville	D Egli 145	29°62'83''S 29°40'59''E	Roadside
Senecio sp. 1	12-11-2014	Camperdown Ingomankulu	D Egli 93	29°76'92''S 30°47'12''E	Grassland
Senecio sp. 2	29-11-2014	Sani Pass Hotel	D Egli 105	29°66'75''S 29°45'83''E	Grassland
Senecio sp. 3	12-02-2015	Howick Umngeni Valley	D Egli 164	29°47'59''S 30°24'67''E	Grassland
Cineraria lyratiformis Cron.	13-02-2014	Nottingham Road	D Egli 63	29°38'60''S 30°03'94''E	Grassland
	29-01-2020	Mooi River	D Egli 233	29°24'77''S 29°99'58''E	Grassland

* Specimens lodged in the NU, but not yet allocated accession numbers.

Table 13. Sites in the KwaZulu-Natal Midlands, South Africa where monthly samples of *Senecio madagascariensis* capitula were taken for capitulum-boring Lepidoptera.

Site name	Co-ordinates (S, E)	Habitat	Altitude (m amsl)	Mean annual rain (mm)*	Mean daily temp (°C)*
Cedara (Hilton)	29°32’22.128” 30°16’05.732”	Grassland/ paddock	1068	885	16.3
Groundcover (Curry’s Post)	29°23’19.046” 30°10’31.065”	Grassland/ paddock	1280	985	15.8
Ukulinga (Mkhondeni)	29°39’44.291” 30°24’18.215”	Grassland/ paddock	759	738	18.4
Raptor Centre (Ashburton)	29°40’33.254” 30°30’42.987”	Dry savannah/ roadside	787	738	18.4

* Data provided by F.J. Mitchell and R.D. Chapman (Department of Natural Resources, KwaZulu-Natal Department of Agriculture and Rural Development).

Table 14. Accession information of sequenced capitulum-feeding lepidopterans collected from *Senecio* species between 2014 and 2020.

Sample ID	BOLD Accession number	BIN	Family	Life Stage	Host Plant	Site	Sampling Date	Latitude	Longitude
Lep001	SABCI514-20	7	Tortricidae	Larva	*Senecio sp. nr conrathii*	Curry's Post, Ground Cover Leather Co.	16-Jan-18	-29.3886	30.17507
Lep002	SABCI515-20	8	Tortricidae	Larva	*Senecio inaequidens*	Drakensburg, Lower Lotheni Road	20-Feb-18	-29.4525	29.76528
Lep003	SABCI516-20	8	Tortricidae	Larva	*Senecio inornatus*	Drakensburg, Lower Lotheni Road	20-Feb-18	-29.6463	29.54611
Lep005	SABCI518-20	6	Tortricidae	Larva	*Senecio affinis*	Cumberland Nature Reserve	27-Mar-18	-29.5187	30.51451
Lep006	SABCI519-20	6	Tortricidae	Larva	*Senecio affinis*	Cumberland Nature Reserve	27-Mar-18	-29.5187	30.51451
Lep009	SABCI522-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep010	SABCI523-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep011	SABCI524-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep012	SABCI525-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep013	SABCI526-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep014	SABCI527-20	1	Pyralidae	Larva	*Senecio inaequidens*	Mbona Private Nature Reserve	21-Oct-14	-29.2796	30.35602
Lep016	SABCI529-20	1	Pyralidae	Larva	*Senecio polyanthemoides*	Curry's Post, Mount Gilboa	12-Dec-19	-29.2705	30.28937
Lep017	SABCI530-20	5	Tortricidae	Larva	*Senecio madagascariensis*	Mpophomeni, Midmar Dam	02-Feb-20	-29.5470	30.18249
Lep018	SABCI531-20	1	Pyralidae	Larva	*Senecio sp.*	Ashburton, African Bird of Prey Sanctuary	28-Oct-19	-29.6793	30.50843
Lep019	SABCI532-20	1	Pyralidae	Larva	*Senecio sp.*	Ashburton, African Bird of Prey Sanctuary	28-Oct-19	-29.6793	30.50843
Lep021	SABCI534-20	1	Pyralidae	Larva	*Senecio sp.*	Ashburton, African Bird of Prey Sanctuary	28-Oct-19	-29.6793	30.50843
Lep022	SABCI535-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-Apr-17	-29.6793	30.50843
Lep023	SABCI536-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-Apr-17	-29.6793	30.50843
Lep024	SABCI537-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-Apr-17	-29.6793	30.50843
Lep028	SABCI540-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	06-Apr-17	-29.5396	30.26803
Lep029	SABCI541-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	06-Apr-17	-29.5396	30.26803
Lep030	SABCI542-20	5	Tortricidae	Larva	*Senecio madagascariensis*	Curry's Post, Ground Cover Leather Co.	06-Apr-17	-29.3886	30.17507
Lep031	SABCI543-20	5	Tortricidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-May-17	-29.6793	30.50843
Lep032	SABCI544-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-May-17	-29.6793	30.50843
Lep033	SABCI545-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	04-May-17	-29.6793	30.50843
Lep034	SABCI539-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Curry's Post, Ground Cover Leather Co.	02-May-17	-29.3886	30.17507
Lep035	SABCI546-20	5	Tortricidae	Larva	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	06-Jun-17	-29.5396	30.26803
Lep036	SABCI547-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Pietermaritzburg, Ukulinga Research Farm	03-Aug-17	-29.6622	30.40488

Lep037	SABCI548-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Pietermaritzburg, Ukulinga Research Farm	07-Sep-17	-29.6622	30.40488
Lep038	SABCI549-20	1	Pyralidae	Larva	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	02-Oct-17	-29.5396	30.26803
Lep039	SABCI550-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Curry’s Post, Ground Cover Leather Co.	18-Feb-18	-29.3886	30.17507
Lep043	SABCI554-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	13-Mar-18	-29.5396	30.26803
Lep044	SABCI555-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Curry’s Post, Ground Cover Leather Co.	13-Mar-18	-29.3886	30.17507
Lep045	SABCI556-20	4	Pterophoridae	Larva	*Senecio madagascariensis*	Curry’s Post, Ground Cover Leather Co.	13-Mar-18	-29.3886	30.17507
Lep047	SABCI558-20	4	Pterophoridae	Larva	*Senecio polyodon*	Karkloof, Karkloof Conservation Centre	02-Dec-14	-29.3462	30.29216
Lep048	SABCI559-20	4	Pterophoridae	Larva	*Senecio polyodon*	Karkloof, Karkloof Conservation Centre	02-Dec-14	-29.3462	30.29216
Lep049	SABCI560-20	4	Pterophoridae	Adult	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	07-Nov-17	-29.5396	30.26803
Lep050	SABCI561-20	4	Pterophoridae	Adult	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	13-Feb-18	-29.5396	30.26803
Lep051	SABCI562-20	4	Pterophoridae	Adult	*Senecio madagascariensis*	Hilton, Cedara College of Agriculture	13-Feb-18	-29.5396	30.26803
Lep052	SABCI563-20	1	Pyralidae	Adult	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	12-Sep-17	-29.6793	30.50843
Lep055	SABCI564-20	1	Pyralidae	Adult	*Senecio madagascariensis*	Pietermaritzburg, Ukulinga Research Farm	02-Oct-17	-29.6622	30.40488
Lep056	SABCI565-20	1	Pyralidae	Adult	*Senecio madagascariensis*	Mooi River	12-Dec-19	-29.2472	29.99796
Lep057	SABCI566-20	1	Pyralidae	Adult	*Senecio madagascariensis*	Ashburton, African Bird of Prey Sanctuary	06-Jul-17	-29.6793	30.50843
Lep058	SABCI567-20	1	Pyralidae	Adult	*Senecio madagascariensis*	Pietermaritzburg, Ukulinga Research Farm	02-Oct-17	-29.6622	30.40488
Lep060	SABCI568-20	2	Plutellidae	Adult	*Senecio madagascariensis*	UKZN Botanical Gardens	08-Jul-20	-29.6252	30.40404
Lep061	SABCI569-20	2	Plutellidae	Adult	*Senecio madagascariensis*	UKZN Botanical Gardens	08-Jul-20	-29.6252	30.40404
Lep062	SABCI570-20	2	Plutellidae	Adult	*Senecio madagascariensis*	UKZN Botanical Gardens	08-Jul-20	-29.6252	30.40404
Lep063	SABCI571-20	3	Tortricidae	Adult	*Senecio madagascariensis*	UKZN Botanical Gardens	08-Jul-20	-29.6252	30.40404

Table 15. Plant species sampled for capitulum-boring lepidopteran larvae from which COI sequences were obtained for host-range analyses.

Species	No. sites*	No. plants	No. capitula	No. larvae
Senecio adnatus DC.	5	22	1088	0
Senecio affinis DC.	4	11	506	2
Senecio bupleuroides DC.	4	9	278	0 (1)
Senecio brevidentatus M.D.Hend	1	1	3	0
Senecio conrathii N.E.Br	2	9	170	0
Senecio coronatus (Thumb.) Harv.	4	23	273	0
Senecio decurrens DC.	1	9	50	0
Senecio glaberrimus DC.	5	23	740	0 (2)
Senecio harveianus MacOwan	-	-	-	- (1)
Senecio heliopsis Hilliard & B.L. Burtt	1	1	67	0
Senecio humidanus C.Jeffrey	2	2	96	0 (1)
Senecio inaequidens DC.	4	18	354	7
Senecio inornatus DC.	6	31	1511	1
Senecio isatidioides Phill. & Sm.	2	10	637	0
Senecio madagascariensis Poir.	11	89	3002	31 (5)
Senecio oxyriifolius DC.	2	12	386	0 (4)
Senecio panduriformis Hilliard	1	5	198	0
Senecio polyanthemoides Sch. Bip.	11	43	2150	1
Senecio polyodon DC.	1	10	281	2
Senecio retrorsus DC.	3	9	309	0
Senecio rhomboideus Harv.	1	1	42	0
Senecio scitus Hutch. & Burtt Davy	3	3	105	0
Senecio scoparius Harv.	1	3	196	0
Senecio serratuloides DC.	2	12	741	0
Senecio skirrhodon DC.	3	8	123	0
Senecio sp. nr. *adnatus* DC.	2	11	915	0
Senecio sp. nr. *adnatus* 2	3	5	342	0
Senecio sp. nr. *affinis* DC.	1	6	325	0
Senecio sp. nr. *conrathii* N.E.Br	2	11	118	1
Senecio sp. nr. *hastatus* L.	1	11	48	0
Senecio sp. nr. *serratuloides* DC.	2	10	418	0
Senecio striatifolius DC.	1	10	74	0
Senecio urophyllus Conrath.	1	1	142	0
Senecio sp. 1	1	12	69	0
Senecio sp. 2	1	4	139	0
Senecio sp. 3	1	5	94	3
Cineraria lyratiformis Cron.	2	15	305	0

* Voucher specimens of plants collected at each site are lodged in the University of KwaZulu-Natal John Bews Herbarium (NU). See Table 3.1 for site details.

** Numbers in brackets denote additional COI sequences from an earlier survey (Egli et al. 2020) that were included in the analyses.

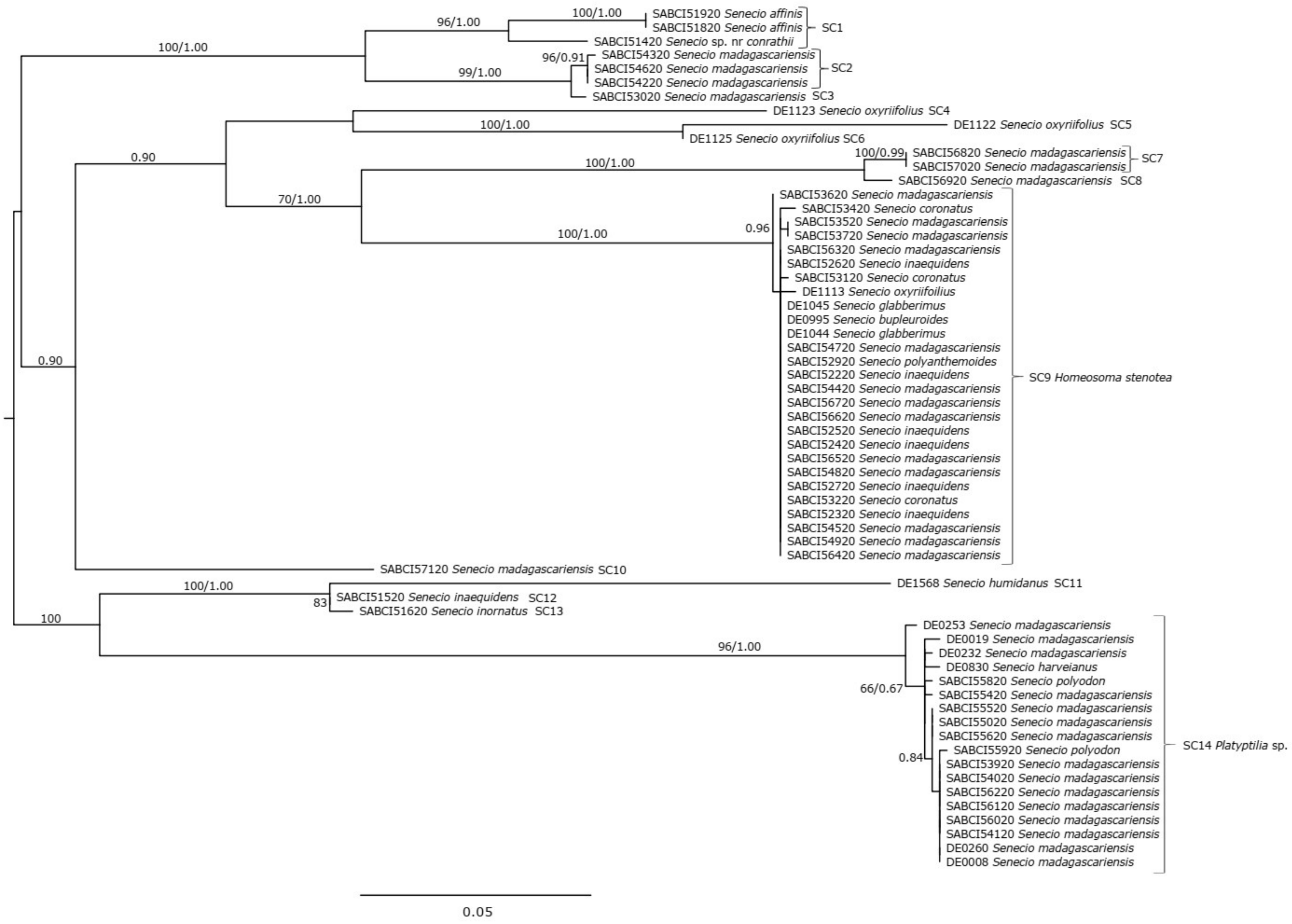


Figure 30. Mid-point rooted maximum likelihood COI phylogeny for capitulum-boring Lepidoptera. Branch values represent bootstrap values and Bayesian posterior probabilities. Only bootstrap values greater than 65% and probability values greater than 0.65 are presented. Branch lengths are proportional to the number of substitutions, which are indicated by the scale bar. *Senecio* host plants are indicated at the end of the branches. Different species clusters (SCs) indicate different lineages (putative species).

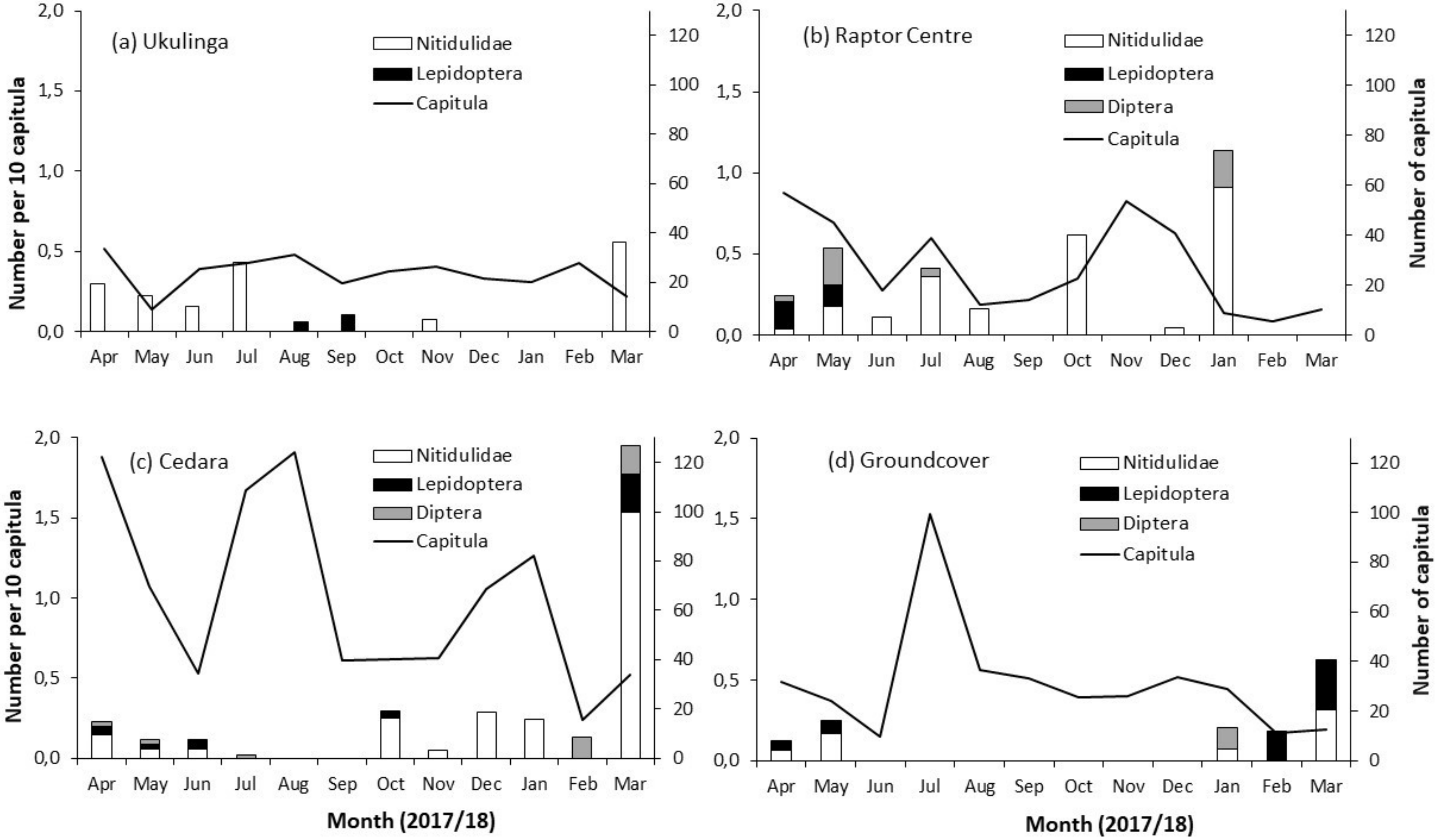


Figure 31. Total numbers of capitulum-boring insect larvae (per 10 capitula) recovered monthly from five *Senecio madagascariensis* plants sampled per month during 2017/18 at lower altitude (a-b) and higher altitude (c-d) sites in KwaZulu-Natal, South Africa. Lines indicate the mean numbers of capitula per plant sampled monthly.

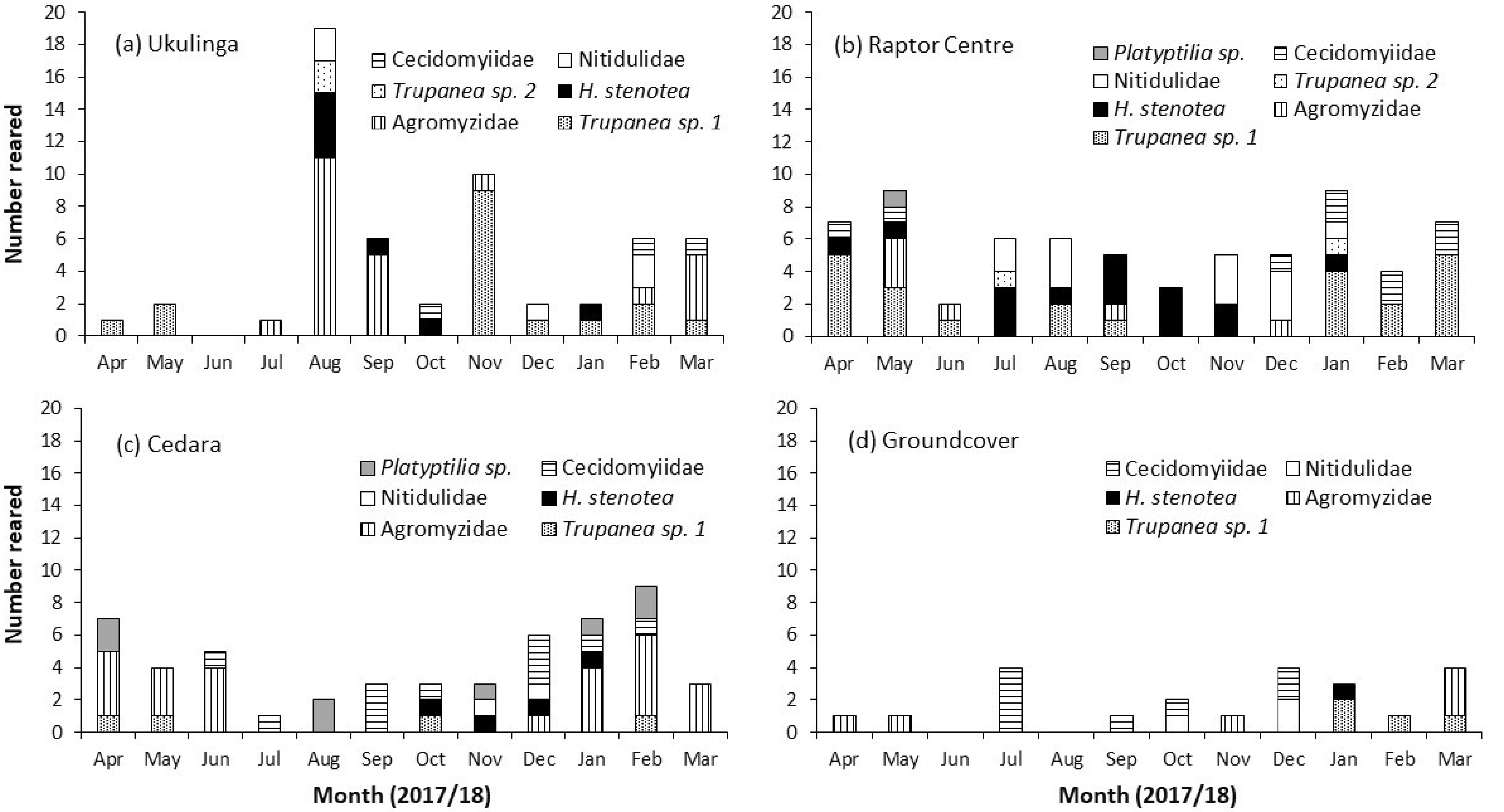


Figure 32. Total numbers of adult insects reared from the capitula of three *Senecio madagascariensis* plants sampled during 2017/18 at lower altitude (a-b) and higher altitude (c-d) sites in KwaZulu-Natal, South Africa. Lepidopteran adults are represented by *Homeosoma stenotea* (black shading) and *Platyptilia* sp. (grey shading).

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