

Molecular and morphometric assessment of the taxonomic status of *Ectemnorhinus* weevil species (Coleoptera: Curculionidae) from the sub-Antarctic Prince Edward Islands

Abstract There are long-standing controversies on the taxonomic status of *Ectemnorhinus* weevil species occurring on the sub-Antarctic Prince Edward Islands. Since the two islands that constitute the Prince Edward Islands archipelago, Marion Island and Prince Edward Island differ in terms of alien invasive species and conservation management strategies, it is important to consider inter-island dynamics when investigating inter-specific relationships. Using a combined molecular phylogenetic and morphometric approach, we attempted to resolve the taxonomic status of the Prince Edward Islands archipelago *Ectemnorhinus* weevil species. A COI gene phylogeny was inferred following genetic characterisation of 52 *Ectemnorhinus* weevils from both islands, and morphometric assessment using a set of 15 linear, external measurements was used to differentiate between the two currently recognised species, *E. similis* and *E. marioni*. Analyses revealed the presence of two genetically and morphometrically distinct species on Prince Edward Island, whilst evidence for a single species, comprising diverse genetically discrete populations was found on Marion Island.

Key words: Weevils, *Ectemnorhinus*, Prince Edward Islands, COI gene, phylogenetics, morphometrics, conservation

Running title: Species status of *Ectemnorhinus* weevils on Marion and Prince Edward Island

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Introduction

The weevils of the South Indian Ocean province of the Southern Ocean all belong to a single, monophyletic unit, the *Ectemnorhinus*-group (Kuschel and Chown 1995). There are approximately 36 species in the group, and they have proven to be taxonomically difficult (Brown 1964; Kuschel 1970; Dreux and Voisin 1989; Chown 1991). In particular, the taxonomic status of *Ectemnorhinus marioni* and *E. similis* from the sub-Antarctic Prince Edward Islands has long been controversial. *Ectemnorhinus similis* was the first *Ectemnorhinus* species described from Marion Island by Waterhouse (1885). Subsequently, Jeannel (1940) described *E. marioni*, which was distinguished from *E. similis* on the basis of the form of the humeri, and interstitial and striae morphology. However, Kuschel (1971) synonymised the two species due to lack of consistent differences in either internal or external morphology. Subsequently, Dreux and Voisin (1986) continued to recognise the two species, noting that they differed by virtue of the form of their elytral striae and interstriae, and the elytral punctuation.

Crafford et al. (1986) recognised three distinct ecotypes within *E. similis* based on body size and colour. Following a detailed investigation of habitat use, feeding biology, life history, morphology and mating preferences, Chown (1990), however, noted that the use of vestiture colour and body length to distinguish between ecotypes was not justified. Rather, he argued that the species complex should be separated into two morphologically similar, but ecologically distinct species. Small-sized (3.77 – 7.79 mm) bryophyte-feeding individuals associated with *Azorella selago*, *Agrostis magellanica*, *Campylopus spp.* and *Ptychomnion ringianum* mires as well as diverse bryophytes including *Ditrichum strictum*, were designated *E. marioni* (Chown 1990). The larger (4.51 – 8.69 mm) angiosperm-feeding individuals associated with *Acaena magellanica* herbfields, *Callitriche antarctica*, *Pringlea antiscorbutica* and *A. selago* were designated *E. similis*. Although *E. similis* feeds mainly on angiosperms, bryophytes, and other cryptogams are incorporated into their diet at the end of the growing season when vascular plant foliage deteriorates (Chown 1989; Chown and Scholtz 1989; Chown 1990). Both *E. marioni* and *E. similis* can be found on *A. selago*, but the former species feeds only on epiphytic bryophytes growing on this plant species (Chown and Scholtz 1989), whereas the latter species feeds both on the *A. selago* and on epiphytic species including the grass, *A. magellanica* and bryophytes.

Apart from variation in body size and diet, *E. marioni* and *E. similis* also differ in the length of their life cycles and times of emergence (Chown 1990). *Ectemnorhinus marioni* exhibits a shorter life cycle with fewer instars and adults are present throughout the year, while

adults of *E. similis* only emerge during summer months, and their emergence appears to be synchronised with the first flushes of angiosperm growth and flowering. Apart from body size, there are neither consistent differences in the male genitalia (Chown 1990), nor consistent differences in either the ovipositor or the spermatheca in females of the two species. Chown (1990) suggested that *E. marioni* and *E. similis* evolved sympatrically in a manner similar to that proposed by Rice (1984), with reproductive isolation being induced by size-based assortative mating associated with dissimilarities in food preference (Chown 1990; Crafford and Chown 1991). This interpretation was subsequently accepted and a wide range of studies have been based on this taxonomy (reviewed in Chown et al. 2002; Klok and Chown 2003), such that management decisions regarding invertebrates on the Prince Edward Islands have been based on this classification (Anonymous 1996).

In addition to a variable taxonomic history, factors influencing both size and population density of *Ectemnorhinus* species on the Prince Edward Islands have also been documented. While the house mouse (*Mus musculus*, sensu lato) was introduced by sealers on Marion Island, the larger of the two Prince Edward Islands, more than 180 years ago (Watkins and Cooper 1986), the smaller Prince Edward Island has remained mouse-free. Mice feed on a variety of invertebrates and plants on the islands, and especially weevils (Gleeson and van Rensburg 1982; Smith et al. 2002). Moreover, temperature in the sub-Antarctic has increased by approximately 1°C in the last 50 years (Smith and Steenkamp 1990), and is believed to have led to an increase in the survival rate of mice during winter months, resulting in an overall population increase (Smith and Steenkamp 1990; Smith 2002). The mean volume contribution of weevil adults found in the guts of mice increased from 7% in 1979/1980 (Gleeson and van Rensburg 1982) to 11% in 1992/1993 (Smith et al. 2002). House mice are thus considered to be responsible for the significant change in the populations of *Ectemnorhinus* species on Marion Island, amounting to almost an order of magnitude decline in biomass between 1976 and 1996 (Chown et al. 2002), and a pronounced difference between population densities on Marion and Prince Edward Islands (Crafford and Scholtz 1987).

Mice are also thought to have caused a reduction in body size of weevil species on Marion Island relative to Prince Edward Island (Chown and Smith 1993). As part of their investigation, the authors noted that the frequency distributions of the size of the *Ectemnorhinus* species differed considerably between the islands. They noted that the situation across the two islands seemed to reflect predation by mice, but also concluded that further investigations were necessary, especially because the genus is taxonomically difficult (see also Chown 1991).

Given the severity of weevil predation on Marion Island and uncertainties on the taxonomic status of the *Ectemnorhinus* species on both islands (Chown 1990, 1991), it is not

clear whether the Prince Edward Island populations alone are sufficient to ensure conservation of the two *Ectemnorhinus* weevil species, especially if predictions that mice predation is likely to continue escalating (Chown et al. 2002) are realised. If the species occur on both islands and if the populations are not so dissimilar that they should be considered different management units, then the current management regime (Anonymous 1996) will suffice. An essential assumption of this regime is that Prince Edward Island serves largely as a mouse-free haven for both islands (but see contrary views in Gremmen and Smith 1999; Chown et al. 2002). However, if the species or populations differ, then management practices would have to change and serious consideration would have to be given to eradication of the mice on Marion Island. Thus, it is clear that resolving the status of the two currently recognised *Ectemnorhinus* species is important from a conservation perspective.

The aim of the present study was, therefore, to evaluate the current taxonomic status of the *Ectemnorhinus* weevil species occurring on the Prince Edward Islands using both molecular and morphometric techniques. The COI gene, that has been successfully used to differentiate between island-bound Coleopteran species (Emerson et al. 1999; Sequeira et al. 2000; Trewick 2000; Caccone and Sbordoni 2001), was selected for genetic characterisation, whilst a set of 15 linear external measurements (Janse van Rensburg et al. 2003) was used for the morphometric assessment.

Materials and methods

Study area and samples

Ectemnorhinus weevil specimens were collected over three consecutive years (April 2001 – April 2003) from 28 localities (Fig. 1) on Marion Island. Samples from Prince Edward Island which has restricted access were collected along an altitudinal gradient (0 – 675 m a.s.l.) at 200 m intervals and from an additional locality consisting largely of *Ditrichum strictum*, in April 2003. Coordinates for all the sampling localities are summarised in Table 1. All specimens were collected by hand and preserved in absolute ethanol.

For the morphometric component of the study, between five and 30 *Ectemnorhinus* specimens per locality were measured. Due to uncertainty about the taxonomic status of *Ectemnorhinus* species on both Marion Island and Prince Edward Island, individuals were only identified as belonging to the genus, based on the generic descriptions provided by Kuschel and Chown (1995), with *a priori* rather than *a posteriori* multivariate morphometric analyses being used to define phenetic groupings.

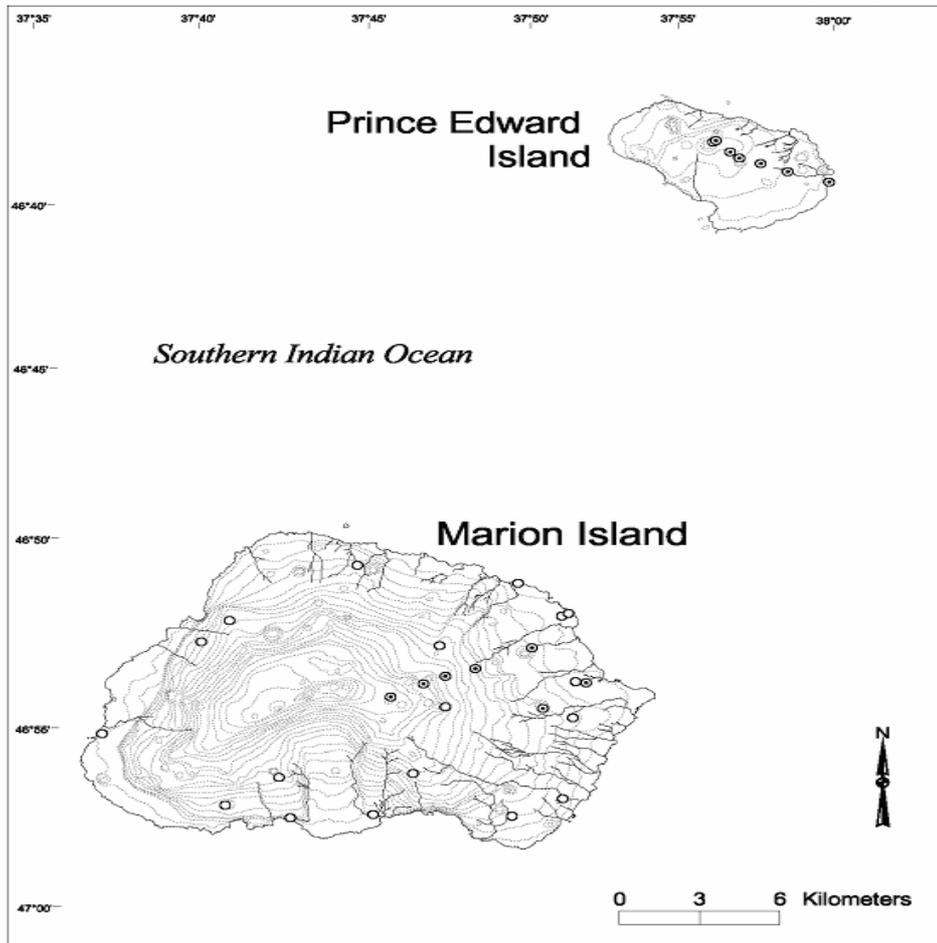


Fig. 1 Map indicating *Ectemnorhinus* weevil sampling localities on Marion Island and Prince Edward Island that correspond to the coordinates summarised in Table 1. Samples collected from all localities were included in the morphometric analyses while only samples from the dotted localities were included in the genetic analyses

Table 1 Summary of sampling locality coordinates for Marion Island (MI) and Prince Edward Island (PE)

| Sampling Locality | Coordinates |
|---|---------------------------|
| MI 200 m Junior's Kop | S 46°52.794' E 37°50.083' |
| MI 400 m First Red Hill | S 46°53.412' E 37°48.21' |
| MI 600 m First Red Hill | S 46°53.647' E 37°47.208' |
| MI 800 m Katedraalkrans | S 46°53.896' E 37°46.482' |
| MI 1000 m | S 46°54.29' E 37°45.375' |
| MI Tate's Hill <i>Pringlea antiscorbutica</i> | S 46°54.6' E 37°50.478' |
| MI Albatross Lakes | S 46°53.82' E 37°51.916' |
| PE Cave Bay 0 m | S 46°38.752' E 37°59.780' |
| PE 200 m | S 46°38.457' E 37°58.396' |
| PE 400 m | S 46°38.211' E 37°57.482' |
| PE 600 m | S 46°37.533' E 37°55.985' |
| PE TvZB 672 m | S 46°37.590' E 37°55.891' |
| PE <i>Ditrichum strictum</i> | S 46°38.057' E 37°56.771' |

*TvZB indicates samples collected at the top of Van Zinderen Bakker Peak

Fifty-two *Ectemnorhinus* individuals from seven localities on both Marion and Prince Edward Islands (Fig. 1) were analysed for the molecular component. In an attempt to ensure adequate representation of species-associated feeding preferences as indicated by Chown (1990), *Ectemnorhinus* individuals were collected from *D. strictum*, *A. selago*, and *P. antiscorbutica*. Individuals collected from *D. strictum* on Marion Island were collected from an *A. selago*-free polar desert site near Albatross Lakes while those collected from *D. strictum* on Prince Edward Island were collected from a site comprising mainly of *D. strictum* (Coordinates given Table 1). As gut contents were not evaluated, we were, however mindful that sampling from a particular plant species did not necessarily imply feeding preference for that plant species. Body size variation, another criterion used by Chown (1990) to distinguish between *E. marioni* and *E. similis*, was accommodated by including the extreme size classes (largest two and the smallest two individuals) per locality. *A priori* assignment into different species was not taken into account in subsequent molecular analyses. *Ectemnorhinus viridis* (Waterhouse 1853) from Heard Island was selected as an outgroup, since it is reasonably closely related to *E. marioni* (Kuschel and Chown 1995).

Molecular characterisation

Following rehydration of ethanol-stored weevils with water, one to two weevil legs per specimen were frozen in liquid nitrogen before being ground and mixed with phosphate-buffered saline (PBS). DNA was extracted using a modified guanidinium thiocyanate (GuSCN)/silica-based method (Boom et al. 1990).

Published primers C1-J-1718 and TL2-N-3014 (Simon et al. 1994) were initially used to generate partial sequence data for representatives of all six weevil species occurring on Marion Island namely, *Bothrometopus elongatus* (Jeannel 1953), *Bothrometopus parvulus* (Waterhouse 1885), *Bothrometopus randi* (Jeannel 1953), *E. marioni* (Jeannel 1940), *E. similis* (Waterhouse 1885) and *Palirhoeus eatoni* (Waterhouse 1879). As these primers generally delivered poor quality sequences, two Marion Island weevil-specific COI primers were designed from the aligned partial sequences, following the guidelines of Rychlik (1993). These Marion Island weevil-specific COI primers termed GF and GR1 (Table 2) amplified a 1059 bp PCR product under the following conditions: 1×Buffer, 0.2 mM dNTP, 0.4 μM of each primer and 1 U *Taq* polymerase in a final volume of 50 μl containing 200 ng of template DNA. A typical temperature profile consisted of an initial denaturation step at 94°C for 90 s, followed by 40 cycles at 94°C for 22 s, 46°C for 30 s and 72°C for 1 min. PCR products were purified and DNA sequences were determined by automated cycle sequencing on an ABI PRISM™ 3100 Analyser using the ABI PRISM Big Dye™ Terminator version 3.0 sequencing standard.

Internal primers termed GF3, GF4, GF5 and GR5 (Table 2) were designed from the sequences initially generated with the Marion Island weevil-specific primers. The latter two primers were used in all subsequent cycle-sequencing reactions to generate a homologous 885 bp region of sequence data. The sequences were viewed and edited in Chromas version 1.43 (McCarthy 1996-1997) and aligned with DAPSA version 4.9 (Harley 2000).

Table 2 List of primers used in this study

| Name | Orientation | Sequence | Tm |
|------------|-------------|---------------------------------|------|
| C1-J-1718 | Forward | 5'GGAGGATTTGGAAATTGATTAGTTCC 3' | 608C |
| TL2-N-3014 | Reverse | 5'ATTATACCGTCTAATCACGTAACCT 3' | 588C |
| GF-1858 | Forward | 5' GGGACAGGTTGAACAGTTTATC 3' | 588C |
| GR1-2938 | Reverse | 5' ATGTTGTTATTCTTGAAGATGAAAG 3' | 548C |
| GF3-2206 | Forward | 5'GGTCACCCAGAAGTATATAT3' | 538C |
| GF4-2662 | Forward | 5'GCTGGAATAGTACAATGATT3' | 538C |
| GF5-1940 | Forward | 5' TACATATAGCAGGTGTATCATC 3' | 548C |
| GR5-2935 | Reverse | 5' GTTATTCTTGAAGATGAAAGATT 3' | 518C |

Tm: Melting temperature, calculated using the formula:

$$Tm = [69.3 + (0.41 * \%GC)] - 650 / \text{primer length}$$

Phylogenetic analyses

Three sequence datasets were compiled, a Marion Island dataset, a Prince Edward Island dataset and a combined Prince Edward Islands archipelago dataset. Neighbor Joining (NJ; Saitou and Nei 1987) and Minimum Evolution (ME; Rzhetsky and Nei 1992) algorithms in MEGA version 2 (Kumar et al. 2001) were used to construct phylogenies with nodal support being assessed by 10 000 bootstrap replications.

Model Test version 3.06 (Posada and Crandall 1998) was used to identify the model of evolution that best fits the data with parameters identified under the Akaike Information Criterion (Akaike 1974) being used for subsequent Maximum Likelihood analyses (Felsenstein 1981). In each case, the TrN + I model with equal rates for all sites that correspond to the General time-reversible model, GTR + I (Rodriguez et al. 1990) was selected. The proportion of invariable sites (I) and three different substitution types estimated for each dataset was as follows: 1) Combined data set: I = 0.7935, rate [A-G] = 48.19, rate [C-T] = 11.65, and other rates = 1.00; 2) Prince Edward Island dataset: I = 0.8018, rate [A-G] = 40.61, rate [C-T] = 9.03, and other rates = 1.00; and 3) Marion Island dataset: I = 0.7985, rate [A-G] = 50.85, rate [C-T] = 12.11, and other rates = 1.00.

Maximum Likelihood analyses were performed in PAUP* version 4.0b10 for Macintosh (Swofford 1999) assuming the above mentioned model parameters prior to bootstrap re-sampling. Bayesian phylogenetic analyses using MrBayes version 3.0B4 (Huelsenbeck and Ronquist 2001) were performed based on the same models and parameters recovered for each of the respective datasets. Analyses were initiated with random starting trees and run for 500 000 generations with Markov chains sampled every 100 generations. Of the 5000 trees obtained, 2000 were discarded as “burn-in”.

Parsimony analyses performed with PAUP* version 4.0b10 included equal weighting and differential weighting schemes such as character weighting where third base positions were down-weighted to 1, and first base positions were up-weighted to 9.76923; successive weighting (Farris 1969); 6 parameter parsimony on its own and combined with both character weighting and successive weighting (Williams and Fitch 1990).

The equality of evolutionary rates between lineages was tested using the relative rate test (Li and Bousquet 1992) in PHYLTEST version 2.0 (Kumar 1996). In addition, the likelihood ratio test (Felsenstein 1981, 1988) was performed and log likelihood scores obtained with and without the molecular clock enforced, were compared. Divergence times were calculated from uncorrected pairwise values and calibrated using 2.3% nucleotide sequence divergence per million years based on the arthropod mtDNA survey of Brower (1994). Haplotype diversity (h) and nucleotide diversity (π) were estimated for each island individually in DNASP 3.51 (Rozas and Rozas 1999). Differences in total body lengths of individuals between clades were determined using analysis of variance (ANOVA; Zar 1996) for Prince Edward Island.

Morphometric analyses

Fifteen morphometric measurements were recorded by a single observer (L.J.v.R.) using a stereomicroscope fitted with a calibrated eyepiece micrometer. Measurements, defined and selected based on a morphometric character selection procedure followed by Janse van Rensburg et al. (2003) included: Total body length (TL), pronotum breadth (PB), femur length (FL), interocular distance (O), metacoxal distance (MT), maximum breadth of elytra (EW), length of first three tarsal segments (T3), meso/metacoxal distance (MM), interantennal distance (A), mesocoxal distance (MS), femur breadth (FB), funicle segments 1, 2, and 3 (F1, F2 and F3), and rest of funicle (FR). Measurements were recorded to the nearest 0.05 mm (TL and EW), 0.03 mm (PB and FL), and 0.01 mm (O, A, F1, F2, F3, FR, T3, MS, MT, MM and FB).

For multivariate morphometric analyses, the absence of multivariate sexual dimorphism (Janse van Rensburg et al. 2003) permitted pooling of sexes for subsequent analyses. For Marion Island, a total of 807 individuals from 28 localities, which provided adequate geographical coverage of *Ectemnorhinus* species, were used for morphometric analysis. A total of 240 *Ectemnorhinus* specimens from six localities on Prince Edward Island were analysed. Data screening revealed five outlier specimens, not considered representative of the populations. A re-examination of these specimens revealed outlier values arising from damaged parts, and to avoid the introduction of bias in the sample, they were excluded from subsequent analyses. After determining the absence of multivariate sexual dimorphism using principal components analysis, these datasets were subjected to a randomisation procedure (Manly 1991), where a new dataset with an equal number of individuals as the original dataset were randomly sampled with replacement for each island to assess whether the absence of multivariate sexual dimorphism in the original dataset was significantly different from the randomly selected dataset.

Following Chimimba et al. (1999), sampling localities on Marion and Prince Edward Islands were grouped into a number of computationally manageable geographical subsets to accommodate for the unweighted pair-group arithmetic average (UPGMA) cluster analyses for the Marion Island ($n = 807$) and the combined island ($n = 1047$) data matrices, since the data matrices were too large for simultaneous specimen-level analyses. The results of the individual-level analyses of the geographic subsets facilitated the grouping of locality mean values in subsequent analyses that accommodated entire island data that were similar to the results of the individual-level analyses. The 28 and 24 genetically identified *Ectemnorhinus* individuals from Marion and Prince Edward Islands, respectively, were included in all morphometric analyses to assist in defining phenetically-derived groupings.

Multivariate analyses included principal components analysis (PCA) and unweighted pair-group arithmetic average (UPGMA) cluster analysis to assess whether species could be identified based on morphometric characters (Sneath and Sokal 1973). Canonical variates analysis (CVA) of genetically defined groupings was also undertaken (Pimentel and Smith 1986) in order to define phenetic groups *a posteriori*. The CVA was followed by a multivariate analysis of variance (MANOVA; Zar 1996) in order to test for statistically significant differences between pre-defined groups. UPGMA cluster analysis was based on both Euclidean distances and product-moment correlation coefficients among Operational Taxonomic Units (OTUs; Sneath and Sokal 1973), while PCA was computed from product-moment coefficients among variables (Sneath and Sokal 1973). All statistical procedures were performed using Statistica version 5.5 (Statsoft 1995).

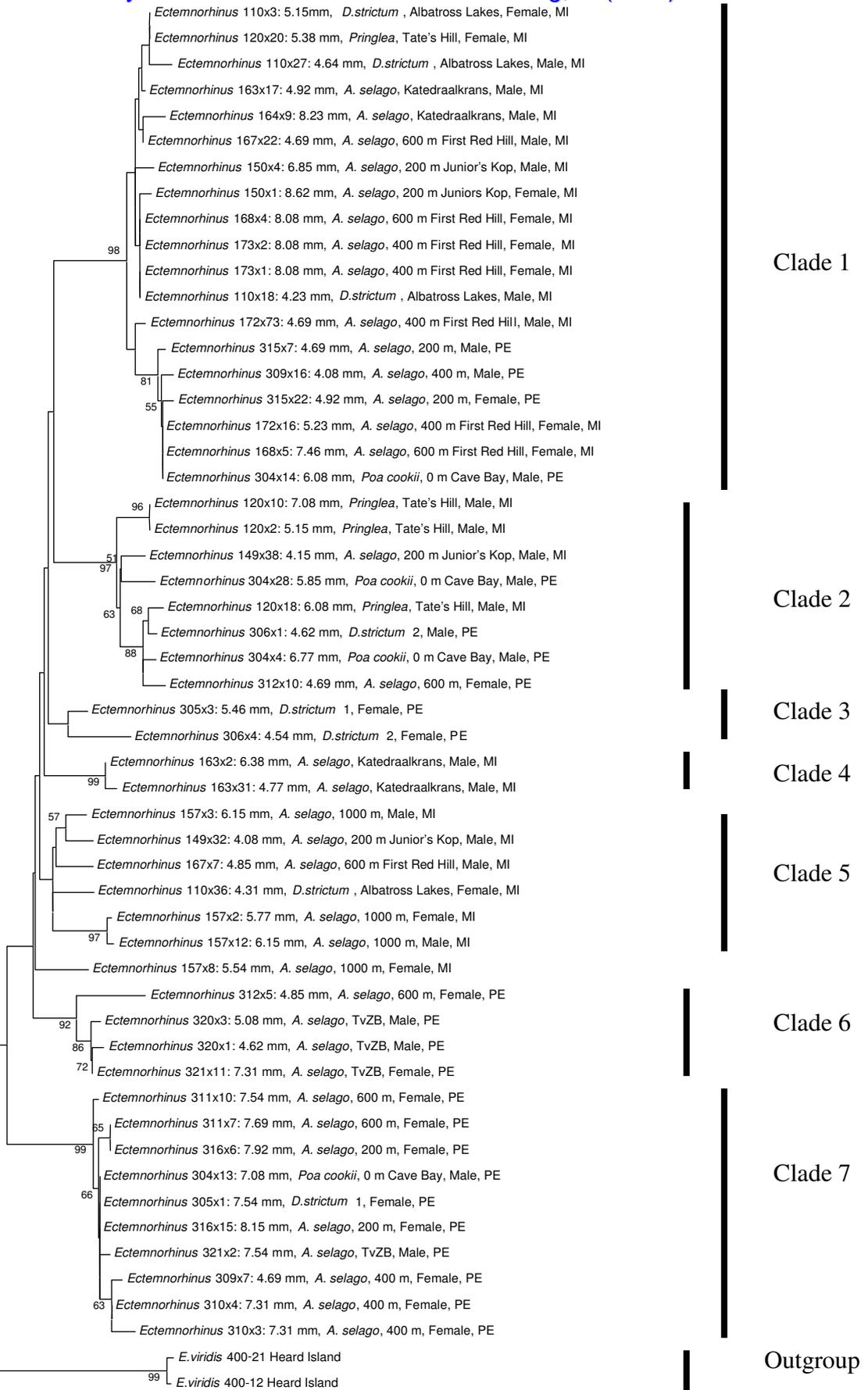
Results

Molecular analyses

A homologous region of 885 bp corresponding to the 5' end of the COI gene was generated for 52 *Ectemnorhinus* individuals and two *E. viridis* outgroup specimens. All sequences have been deposited in the Genbank database under accession numbers AY762267 - AY762320. For the combined dataset, 775 of the 885 sites were conserved across all 54 sequences and 98 of the 142 variable sites were parsimony informative. The % A + T was 68.6% and the transition (ti)/transversion (tv) ratio was 6. Third base position substitutions accounted for 88.2% of the variation and the remaining 11.8% was due to first base substitutions. Mutations at nucleotide level gave rise to five non-synonymous amino acid substitutions at codons 19, 85, 241, 279, and 289. Of the 52 Prince Edward Island archipelago *Ectemnorhinus* individuals sequenced, 35 had unique haplotypes.

When analysing the combined Prince Edward Island archipelago dataset, all major clades (numbered 1-7; Fig. 2a) were consistently recovered across all methods of phylogenetic analysis. Clades 6 and 7 consisted solely of individuals collected on Prince Edward Island, while clades 1 and 2 incorporated individuals from both islands. In clade 1, Marion Island individuals ranging in size from 4.23 mm to 8.08 mm, and collected on *A. selago*, *P. antiscorbutica*, and *D. strictum*, grouped together. Similarly in clade 7, individuals collected on both *A. selago* and *D. strictum* from Prince Edward Island grouped together.

a



0.01

Fig. 2a Minimum Evolution (ME) trees inferred using the Tamura-Nei distance correction algorithm for the combined data set. For each specimen, the sample number is indicated followed by the body length measurement, plant species it was collected from, locality, sex and the island of origin (where 'MI'

denotes Marion Island and 'PE' indicates Prince Edward Island). Nodal support was assessed by 10 000 bootstrap replications, with only those bootstrap values ≥ 50 being indicated next to the relevant nodes. TvZB indicates samples collected at the top of Van Zinderen Bakker Peak at an elevation of 672 m above sea level

When the island datasets were analysed separately, the Marion Island phylogeny revealed that there were 22 unique haplotypes from 28 sequences. Nucleotide diversity π was 0.01217 and haplotype diversity h was 0.976. Four well-supported clades (1, 2, 4, 5) were recovered, but individuals within these clades did not group according to either body size or vegetation type from which individuals were collected (Fig. 2b). The largest between-clade Kimura-2-parameter genetic distance for Marion Island was estimated to be 1.9%.

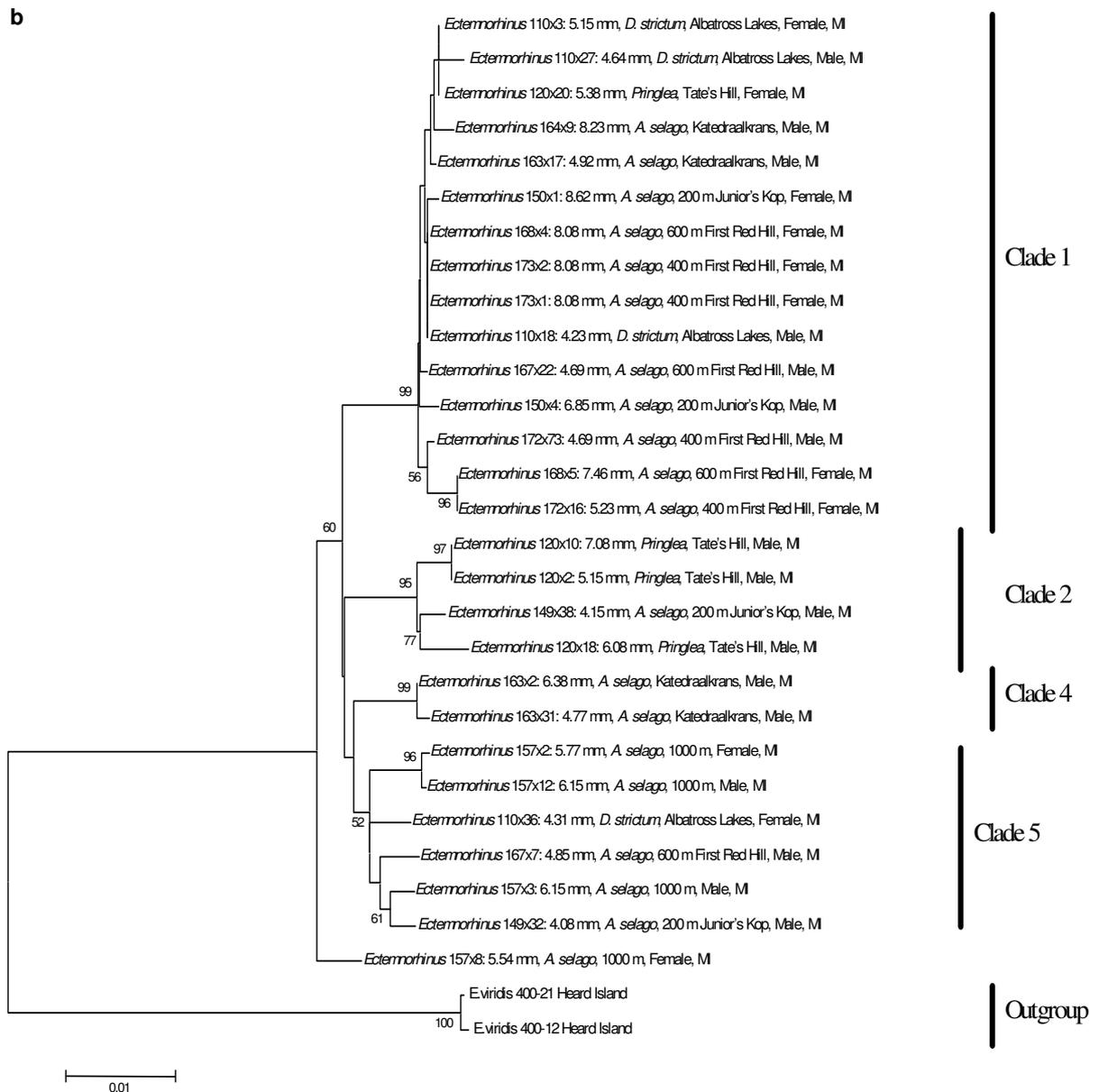


Fig. 2b Minimum Evolution (ME) trees inferred using the Tamura-Nei distance correction algorithm for Marion Island only samples. For each specimen, the sample number is indicated followed by the body length measurement, plant species it was collected from, locality, sex and the island of origin (where 'MI' denotes Marion Island and 'PE' indicates Prince Edward Island). Clades are numbered according to the clades initially identified following analysis of the combined dataset. Nodal support was assessed

by 10 000 bootstrap replications, with only those bootstrap values ≥ 50 being indicated next to the relevant nodes. TvZB indicates samples collected at the top of Van Zinderen Bakker Peak at an elevation of 672 m above sea level

For Prince Edward Island, nucleotide (π) and haplotype diversity (h) was estimated to be = 0.01687 and 0.986, respectively. A large number of unique haplotypes, 21 from 24 sequences, were recovered. Individuals from Prince Edward Island clustered within five distinct clades, of which clades 1, 2, 6, and 7 had high levels ($> 85\%$) of bootstrap support (Fig. 2c).

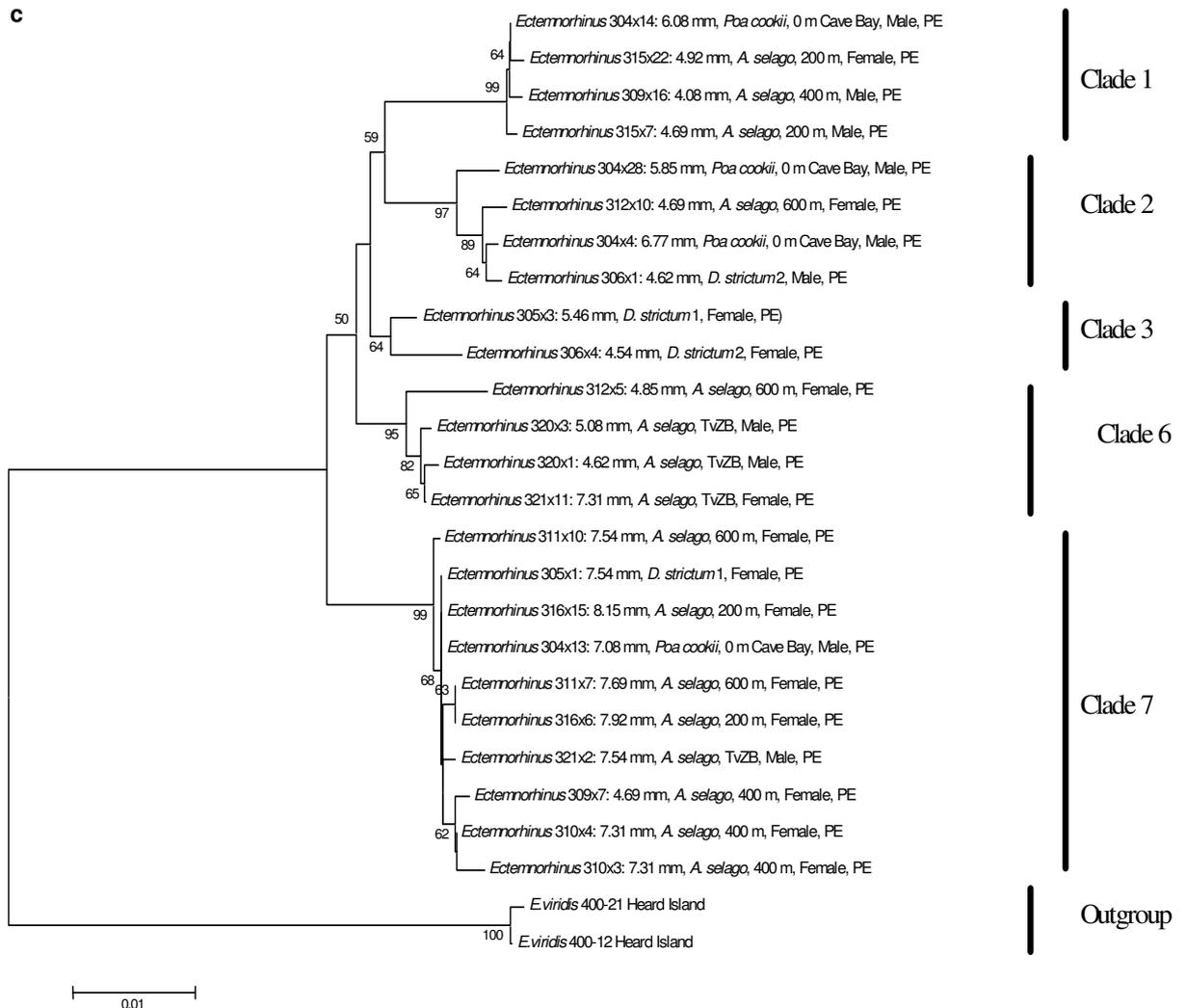


Fig. 2c Minimum Evolution (ME) trees inferred using the Tamura-Nei distance correction algorithm for Prince Edward Island only samples. For each specimen, the sample number is indicated followed by the body length measurement, plant species it was collected from, locality, sex and the island of origin (where 'MI' denotes Marion Island and 'PE' indicates Prince Edward Island). Clades are numbered according to the clades initially identified following analysis of the combined dataset **a**. Nodal support was assessed by 10 000 bootstrap replications, with only those bootstrap values ≥ 50 being indicated next to the relevant nodes. TvZB indicates samples collected at the top of Van Zinderen Bakker Peak at an elevation of 672 m above sea level

Clades 6 and 7 retained their autonomy, even in the combined island analysis (Fig. 2a). The largest between-clade distance was 2.6% and in contrast to Marion Island, clade 7 individuals

were significantly larger than those of the other Prince Edward Island clades (ANOVA: $F = 9.38$, $P < 0.05$). Clade 7 consisted of individuals collected from both *A. selago* and *D. strictum* which indicates that although there is structuring according to body size on Prince Edward Island, there is none according to plant species from which they were collected.

No significant rate heterogeneity was found among the substitution rates at $P < 0.05$ according to both relative rate and likelihood ratio tests. Therefore, it was concluded that *Ectemnorhinus* individuals from the Prince Edward Islands do not evolve at markedly different rates and a molecular clock based on the mtDNA clock for arthropods (Brower 1994) could therefore, be imposed. In consequence, it was estimated that these *Ectemnorhinus* weevils arose approximately 0.49 million years ago (MYA) and 0.33 MYA on Prince Edward and Marion Islands, respectively.

Morphometric analyses

Principal component analyses based on both original and randomly selected data for both Marion and Prince Edward Islands, showed no grouping of the sexes indicating the absence of multivariate morphometric sexual dimorphism within datasets.

All multivariate morphometric analyses of both individual and combined island datasets as well as individual-level analyses and those based on mean values were similar, and are best illustrated by PCA results. The PCA of the combined Prince Edward Island archipelago dataset (encompassing all size classes) showed neither a distinct separation with reference to the two currently recognised *Ectemnorhinus* species nor with regard to island of origin (Fig. 3).

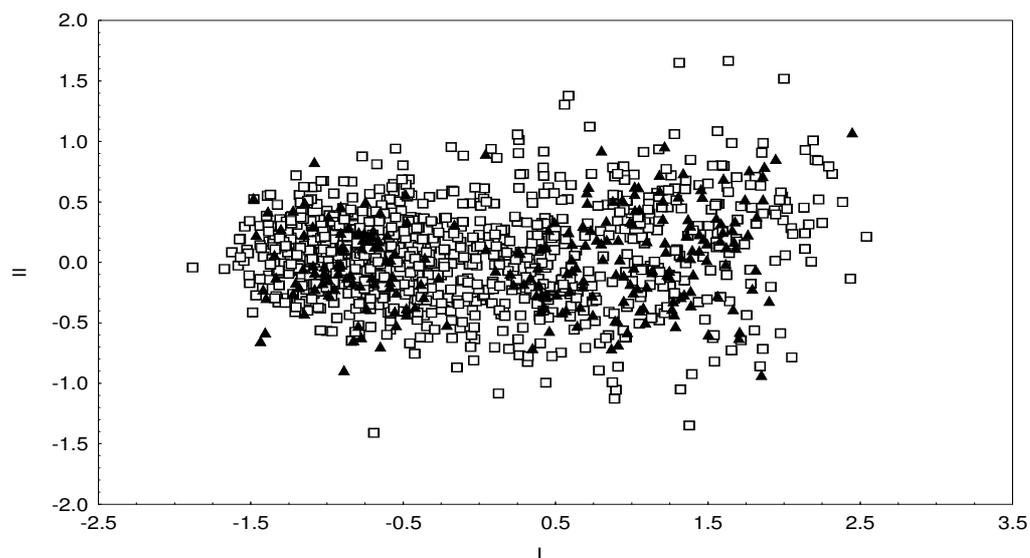


Fig. 3 Components I and II from a principal components analysis of *Ectemnorhinus* species collected from both Marion Island (*open squares*) and Prince Edward Island (*closed triangles*)

Lack of separation, however, seems to be largely confounded by a large degree of body size variation among individuals from the two islands. This is reflected by the high positive loadings of the measurements on the first PCA axis that accounted for 86.08% of the total variance (Table 3).

Table 3 Loadings of variables on components I and II from principal components analyses of pooled samples of *Ectemnorhinus marioni* and *E. similis* from a) Marion Island b) Prince Edward Island and c) Marion and Prince Edward Islands combined

| | a) Marion Island | | b) Prince Edward Island | | c) Marion and Prince Edward Island | |
|----------|----------------------|--------|-------------------------|--------|------------------------------------|--------|
| | Principal components | | Principal components | | Principal components | |
| Variable | I | II | I | II | I | II |
| TL | 0.974 | -0.029 | 0.989 | 0.057 | 0.976 | -0.012 |
| EW | 0.956 | -0.036 | 0.976 | 0.041 | 0.960 | -0.038 |
| PB | 0.946 | -0.042 | 0.986 | 0.080 | 0.952 | -0.066 |
| O | 0.952 | -0.009 | 0.977 | -0.006 | 0.952 | -0.058 |
| A | 0.897 | -0.012 | 0.957 | 0.090 | 0.906 | -0.065 |
| F1 | 0.940 | -0.047 | 0.959 | -0.020 | 0.944 | -0.041 |
| F2 | 0.938 | -0.059 | 0.961 | -0.051 | 0.941 | -0.062 |
| F3 | 0.884 | -0.052 | 0.921 | -0.115 | 0.890 | -0.093 |
| FR | 0.931 | -0.054 | 0.938 | -0.064 | 0.930 | -0.013 |
| T3 | 0.956 | -0.043 | 0.807 | -0.517 | 0.949 | -0.010 |
| MS | 0.555 | 0.830 | 0.833 | 0.258 | 0.712 | 0.698 |
| MT | 0.936 | 0.044 | 0.945 | 0.071 | 0.937 | 0.082 |
| MM | 0.875 | -0.071 | 0.950 | 0.089 | 0.889 | -0.045 |
| FL | 0.986 | -0.061 | 0.988 | 0.020 | 0.986 | -0.054 |
| FB | 0.958 | -0.023 | 0.973 | 0.011 | 0.960 | -0.055 |
| % trace | 84.23 | 4.78 | 89.37 | 2.58 | 86.08 | 3.54 |

* Total body length (TL), pronotum breadth (PB), femur length (FL), interocular distance (O), metacoxal distance (MT), maximum breadth of elytra (EW), length of first three tarsal segments (T3), meso/metacoxal distance (MM), interantennal distance (A), mesocoxal distance (MS), femur breadth (FB), funicle segments (F1, F2 and F3) and rest of funicle (FR)

A PCA restricted to the 52 genetically identified specimens from both Marion and Prince Edward Islands indicated no separation based on either body size or shape in the Marion Island sample. However, two groups are observed for Prince Edward Island *Ectemnorhinus* species, with only a single *Ectemnorhinus* individual from group A (clades 1, 2, 3 and 6: Fig. 2c) falling within *Ectemnorhinus* group B (clade 7: Fig. 2c) (Fig. 4a).

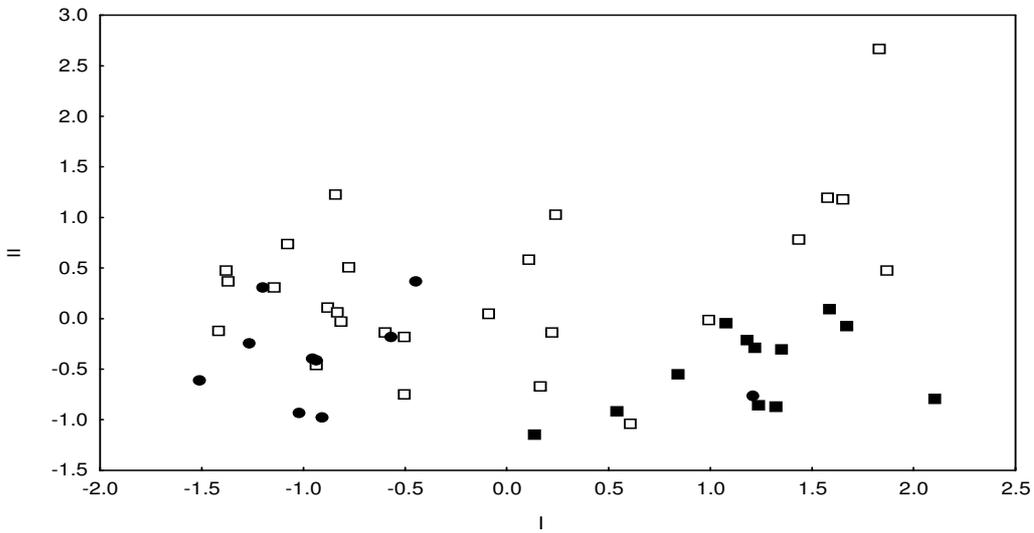


Fig. 4a Components I and II from a principal components analysis of genetically identified individuals from Marion Island (*open squares*) and Prince Edward Island, *Ectemnorhinus* group A (*closed circles*) and *Ectemnorhinus* group B (*closed squares*)

The 52 genetically identified specimens showed a more pronounced and statistically significant separation between *Ectemnorhinus* group A, B and the Marion Island *Ectemnorhinus* specimens on the first CVA axis. A shape related separation was observed on the second CVA axis between the Marion Island *Ectemnorhinus* species and *Ectemnorhinus* group A from Prince Edward Island (Fig. 4b). A MANOVA showed a statistically significant phenetic differences between these pre-defined groupings ($F_{(30,62)} = 4.34, P < 0.0001$).

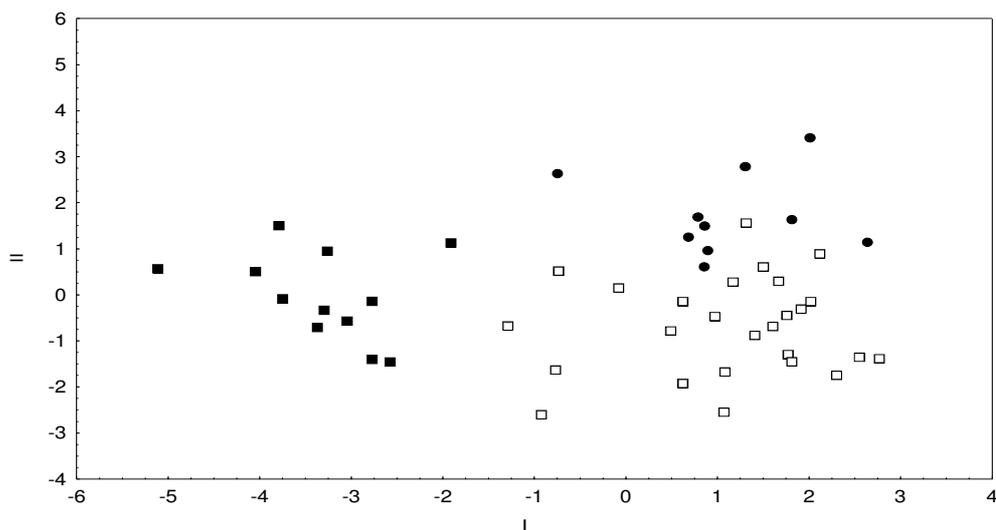


Fig. 4b Components I and II from a canonical variates analysis (CVA) of genetically identified individuals from Marion Island (*open squares*) and Prince Edward Island, *Ectemnorhinus* group A (*closed circles*) and *Ectemnorhinus* group B (*closed squares*)

A PCA of Marion Island samples (represented by all size classes) showed a considerable degree of phenetic variation, but no separation based on either size or shape variables (Fig. 5a).

Principal component I (84.23% of the total variance) had high positive loadings on all measurements (Table 3), highlighting the importance of size variation. No separation was observed on the second (Table 3) and subsequent PCA axes.

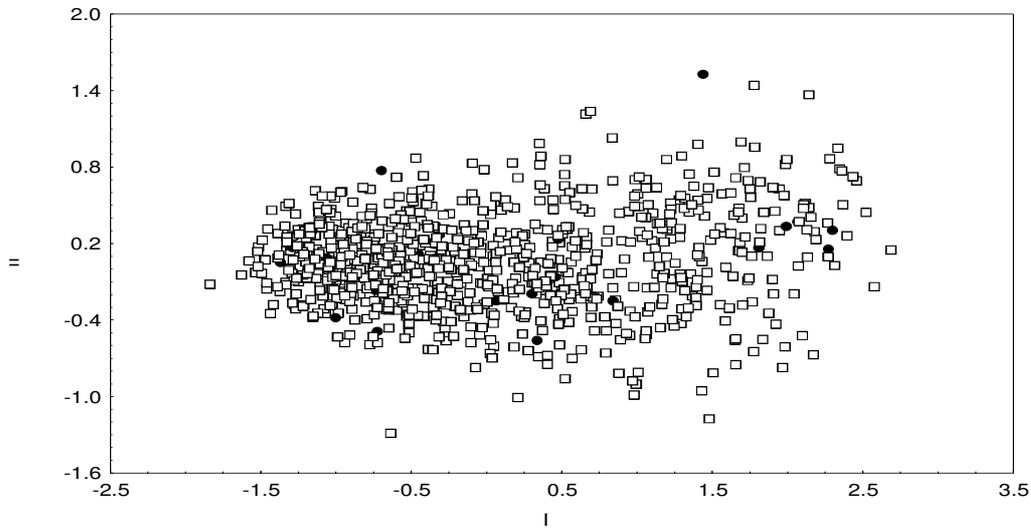


Fig. 5a Components I and II from a principal components analysis of *Ectemnorhinus* species collected from Marion Island. Genetically identified individuals are indicated with black circles

A PCA, comprising all size classes and including genetically identified Prince Edward Island samples (Fig 5b), however, showed two phenetic groupings for the *Ectemnorhinus* species. Only a single genetically-identified individual of *Ectemnorhinus* group A clustered with individuals of *Ectemnorhinus* group B. Separation between the two groups is based on a size- rather than a shape-related variation, as is shown by PCA axis I (89.37%), which has high positive loadings on the measurements (Table 3).

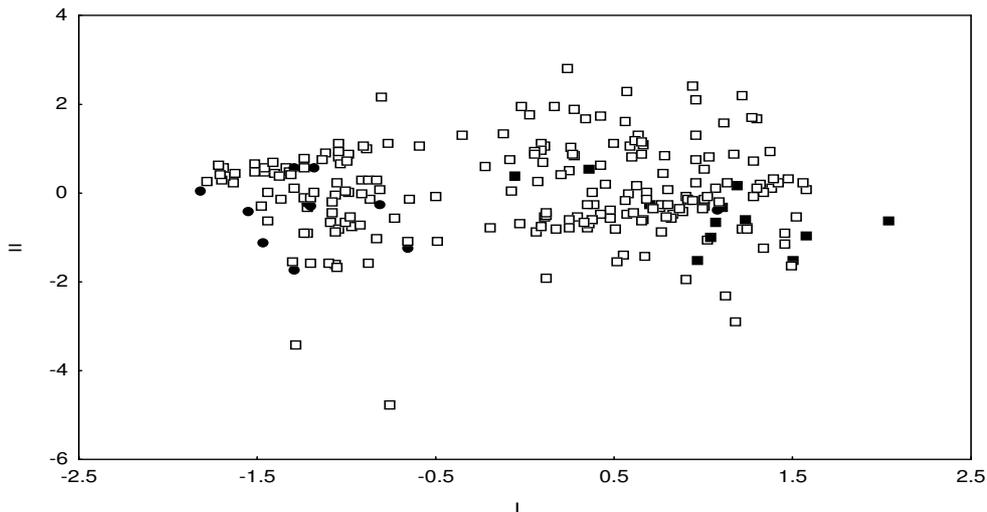


Fig. 5b Components I and II from a principal components analysis of *Ectemnorhinus* species collected from Prince Edward Island. Genetically identified *Ectemnorhinus* group A (closed circles) and *Ectemnorhinus* group B (closed squares) are indicated

Discussion

Assessment of the taxonomic status of weevil species originally described from Marion Island was undertaken using a combined molecular and morphometric approaches. The COI gene phylogeny identified four recently diverged, well-supported clades on Marion Island, but gave no support for the presence of the two species, designated *E. marioni* and *E. similis* by Chown (1990). None of the clades in the molecular phylogeny displayed clustering on the basis of body size or according to plant species from which they were collected. In addition, multivariate analyses of the Marion Island sample showed no separation of *Ectemnorhinus* individuals according to either body size or body shape variation. The molecular analyses, therefore, suggest that previous morphologically- and ecologically-defined distinguishing characteristics (Crafford et al. 1986; Chown and Scholtz 1989; Chown 1990) do not correspond to the clades identified using genetic markers. Moreover, the current morphological analysis once again confirms that size variation is confounding the difference between individuals within the genus (see Brown 1964; Kuschel 1970, 1971; Crafford et al. 1986; Chown and Scholtz 1989; Chown 1990, 1991). In addition, because the morphological analysis was undertaken with individuals that could be assigned to clades based on molecular data, the present analysis also suggests that morphological evolution is much more complex than has previously been considered.

In contrast to the results obtained from Marion Island, two major size-distinct clades were discernible for Prince Edward Island from both the genetic and the morphometric analyses. Sequence divergence values of 2.6% were observed between the two major Prince Edward Island clades that are greater than the intra-generic Kimura-2-parameter genetic distances of 1.5% and 2.1% reported for arthropods from other island systems (Trewick 2000). This, together with the high levels of bootstrap support for the two size- and genetically-distinct clades, suggests that the present recognition of two species distinguishable on the basis of size is supported on Prince Edward Island. The presence of two size-discrete groupings of *Ectemnorhinus* individuals on Prince Edward Island was also confirmed by the morphometric analyses, where all multivariate analyses indicated the presence of two size-related phenetic groupings. The single *Ectemnorhinus* individual from group A, that clustered within the *Ectemnorhinus* group B assemblage may be indicative of the extent of body size variation within *Ectemnorhinus* species.

While our data suggest the presence of two species on Prince Edward Island and only one on Marion Island, both *E. marioni* and *E. similis* were originally described from Marion Island. As *Ectemnorhinus* individuals found on Prince Edward Island were morphologically

and ecologically similar to Marion Island individuals, it was concluded that both species, therefore, also occurred on Prince Edward Island (Dreux 1971). The critical question that may now be posed relates to the apparent disappearance of one species of *Ectemnorhinus* on Marion Island. Was there only one *Ectemnorhinus* species to begin with on Marion Island or is it possible there were indeed two species 65 years ago when Jeannel (1940) first described *E. marioni* as a second species distinct from *E. similis*?

The first hypothesis for the observed difference in weevil assemblage between the two islands is the loss of one of the originally described *Ectemnorhinus* species from Marion Island. One possible cause for the loss could be that the reduction in body size of *E. similis* through size-selective predation by mice (Chown and Smith 1993; Smith et al. 2002) which would have removed the size-induced reproductive barrier that was proposed on grounds of the significant relationship between female and male body size in *in-copula* pairs observed by Chown (1990) on Marion Island. This scenario would allow the two previously recognised species to interbreed. In addition to mice predation, it is also possible that climate change may play a role as temperature on the Prince Edward Islands has increased by an average of 0.04°C per year since the late 1960s (Smith 1991). It is well-known that in arthropods, and indeed most invertebrates, increasing developmental temperatures lead to a decline in body size (Atkinson 1994). Moreover, if temperatures increase to such an extent that generation time is much shorter than season length, then additional declines in body size with increasing temperature can be expected (Kozłowski et al. 2004). Investigations of weevil species on Marion Island have shown that long-term warming (at least since the 1960s) may well have led to on-going declines in body size, accompanied by a secondary, significant influence of mouse predation (L. Janse van Rensburg, unpublished data). These changes in the environment may select for smaller individuals within the populations, leading eventually to introgression.

The second hypothesis is that there was originally only one *Ectemnorhinus* species on Marion Island that was erroneously identified as two species. The original descriptions of *E. similis* by Waterhouse (1885) and *E. marioni* by Jeannel (1940) indicate that there are distinct differences between the two species. The controversial taxonomic status of these two species is, however, a clear indication of the difficulty taxonomists were faced with in the past when using morphological characteristics alone. One possible reason for the different status of *Ectemnorhinus* species between the two islands may also be due to differences in glaciation histories, as Marion Island was extensively glaciated whilst Prince Edward Island was not (Verwoerd 1971). As a result, weevils on Prince Edward Island would have had longer exposure to vascular plants as an additional, more nutritious food source to bryophytes than those on Marion Island giving rise to two species, a smaller one with a preference for bryophytes and a larger one with a preference for angiosperms, as suggested by Chown (1990).

Another possible explanation for the differential island species status may be due to differences in colonisation times. Results from this study indicate that *Ectemnorhinus* weevils colonised Prince Edward Island approximately 0.49 million years ago, soon after the islands emerged, while Marion Island was colonised approximately 0.16 million years later.

Whatever the underlying cause(s), there is no doubt that presently a marked difference in the species between the islands exists. The two islands also differ considerably with respect to the genetic composition of the populations, with each island having numerous island-unique haplotypes and only one haplotype being shared between the islands. Prince Edward Island can thus not be considered as a safe haven for *Ectemnorhinus* populations that are on Marion Island. The *Ectemnorhinus* weevils populations from the two islands are simply too distinct and should be considered different management units. Consequently, the current management regime at the Prince Edward islands should be revised. Moreover, there is an urgent need to explore possibilities of either eradicating or drastically controlling mice on Marion Island as a long-term goal to preserve the genetic variation unique to Marion Island. In the interim, the current policy of restricting human visits to Prince Edward Island should be strictly maintained.

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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein