Patterns and processes underlying evolutionary significant units in the *Platypleura stridula* L. species complex (Hemiptera: Cicadidae) in the Cape Floristic Region, South Africa

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Abstract

Cicadas have been shown to be useful organisms for examining the effects of distribution, plant association and geographical barriers on gene flow between populations. The cicadas of the Platypleura stridula species complex are restricted to the biologically diverse Cape Floristic Region (CFR) of South Africa. They are thus an excellent study group for elucidating the mechanisms by which hemipteran diversity is generated and maintained in the CFR. Phylogeographical analysis of this species complex using mitochondrial DNA Cytochrome Oxidase I (COI) and ribosomal 16S sequence data, coupled with preliminary morphological and acoustic data, resolves six clades, each of which has specific host-plant associations and distinct geographical ranges. The phylogeographical structure implies simultaneous or near-simultaneous radiation events, coupled with shifts in host-plant associations. When calibrated using published COI and 16S substitution rates typical for related insects, these lineages date back to the late Pliocene - early Pleistocene, coincident with vegetation change, altered drainage patterns and accelerated erosion in response to neotectonic crustal uplift and cyclic Pleistocene climate change, and glaciation-associated changes in climate and sea level.

Introduction

Phytophagous insects are diverse, in many cases possessing specialized plant associations as a result of plant chemistry and assortative mating (Feder et al. 1994; Percy et al. 2004). These insects provide tools to test models of speciation (Cooley et al. 2001; Despres et al. 2002), dietary specialization (Morse & Farrell 2005) and host-race formation in the absence of geographical isolation (Simon et al . 2003). Phylogeographical studies can lead to the identification of places and processes central to the origin and maintenance of biological diversity (Bermingham & Moritz 1998; Moritz & Faith 1998; Soltis et al. 2006); factors that are essential for conservation (Kremen et al.1993; Moritz 2002). Furthermore, phylogeographical and phylogenetic studies of phytophagous insects, such as cicadas, can illuminate the effects of past climate change (Buckley et al. 2001) and the origins of extant taxa (Arensburger et al. 2004).

The Cape Floristic Region (CFR) houses a large proportion of southern Africa's unique flora and fauna and is one of the most biodiverse regions known (Goldblatt & Manning 2002; Galley & Linder 2006). Many factors, not all mutually exclusive, have been cited as explanations for the unusually high biodiversity of the CFR. The role of climate change during the Pliocene and Pleistocene has been particularly debated, with support for conflicting hypotheses. Tyson (1999), Richardson et al. (2001) and Cowling et al. (2005) postulate rapid, dramatic climate shifts in southern Africa, while Weaver et al. (1998), Meadows & Baxter (1999), Dynesius & Jansson (2000) and Barraclough (2006) suggest that there was relative stability in the Cape region. The topographical complexity of the region, with an abundance of altitudinal gradients, may have allowed organisms to migrate altitudinally, acting as a buffer to the effects of climate change (Linder & Vlok 1991; Midgley et al. 2001). Furthermore, the region has undergone neotectonic crustal uplift during the Pliocene and Pleistocene by as much as 900 m in the east (Artyushkov & Hofmann 1998), resulting in a westward tilt of the subcontinent. The combination of uplift and rapid sea-level change associated with the Pleistocene glacial cycles would alternately expose and inundate much of

the wide continental shelf, especially south of Cape Agulhas (Dingle & Rogers 1972). These geological and climatic perturbations may underlie speciation in many of the region's plants and animals (Goldblatt 1978; Daniels et al. 2001; Richardson et al. 2001; Gouws et al. 2004; Cowling et al. 2005; Linder 2005; Tolley et al. 2006), and the CFR thus serves as a natural laboratory for studies on speciation. Here we present the results of the first phylogeographical study on a hemipteran from the CFR: the Platypleura stridula L. complex of cicada species. Cicadas are peculiar amongst phytophagous insects in that most of their life cycle is subterranean (Moulds 1990), where they are obligate plant-xylem feeders (Cryan 2005). In general, cicadas are not considered to be vagile (de Boer & Duffels 1996) and commonly possess relatively high rates of endemism and plant specificity (Villet & van Noort 1999). Furthermore, they rely on long-range mate attraction, using loud, species-specific acoustic signals (Claridge 1985; Villet 1995), which would tend to lessen selection pressures leading to the diversification of short-range cues, including those of male genital morphology (Quartau et al . 2000). When coupled with the strong selection for predator avoidance exhibited by cicadas (Villet & van Noort 1999), it is not unusual that there is a propensity to form morphologically cryptic species within the family (Quartau et al. 2000). Thus morphology alone is often insufficient to reveal the relationship between closely related species, and molecular characters provide a useful additional source of data. Although the relative rate of character evolution between nuclear and mitochondrial DNA (mtDNA) is gene-specific, mtDNA data are especially useful when working with recently diverged taxa, due to their higher rate of character evolution relative to nuclear DNA (Moore 1995; Lin & Danforth 2004).

The tribe Platypleurini is distributed from the Cape of South Africa northwards throughout the Afrotropics, via Arabia, India and Southeast Asia, to Korea and Japan (Distant 1906; Duffels & van der Laan 1985; Lee & Hayashi 2003). It is absent from Australia (Moulds 1990), Europe and the New World, implying that the group is not Gondwanan. Previous phylogenetic work on South African species within the genus Platypleura Amyot and Serville, 1843, implied that these cicadas show an ancestral plant association with Acacia species, with speciation sporadically associated with host plant shifts (Villet et al. 2004).

Four species were described in the P. stridula species complex, two of which are currently recognized: P. stridula (Linnaeus, 1758) and P. capensis (Linnaeus, 1764). Cicada catenata Drury, 1773 and C. nigrolinea De Geer, 1773 are currently treated as synonyms of P. stridula (Distant 1906; Duffels & van der Laan 1985; Villet 1989), but the taxonomy of this group is still unresolved. A putative, localized species, Platypleura sp. 10, was recently discovered in the Western Cape, based primarily on songs. These species are abundant and posses a relatively linear north–south (in the case of P. stridula and Platypleura sp. 10) or east–west (in the case of P. capensis) distribution within the CFR (Villet 1989; Villet et al . 2004). All species have moved onto a variety of nonleguminous hosts and appear to have become oligophagous (Villet et al. 2004). Their dietary breadth seems unusual, considering the narrow plant associations of other closely related species within the genus (Villet et al. 2004).

Given its checkered taxonomic history, a previously observed morphological intractability and a distribution restricted to the CFR, this taxon is a good candidate for examining the effects of environmental processes, plant association and geographical barriers to gene flow within a phylum that has been relatively neglected by scientists studying the CFR biota (e.g. Wishart & Hughes 2001; Klass et al. 2003). We test the hypothesis that there are more than two species in this complex, using independent molecular (mtDNA-sequence), morphological and ethological data, and assess the phylogenetic and phylogeographical relationships to infer the causes of this taxonomic diversity.

Methods

Sampling and laboratory protocols

Tymbal muscles were collected in 96% alcohol from one specimen of Platypleura sp. 10, one specimen of Platypleura capensis from each of 66 sites (Table 1), and one specimen of P. stridula from each of 15 sites (Table 2), covering most of their respective geographical ranges. In addition, three other southern African species of Platypleura (Villet et al. 2004) were included as outgroups for the analysis (Table 2). All voucher specimens are housed at the Albany Museum, Grahamstown. Plant-association data were collected with almost every specimen and supplemented with museum data.

Table 1 Locality, host plant, voucher number and GenBank accession no. of samples within each clade of the *Platypleura stridula* complex

Clade	Locality	Latitude	Longitude	Collection #	Host plants	GenBank no. (COI)	GenBank no. (16S)
EC	Gulu River	33°07′04.2″ S	27°43′31.7″ E	MHV 063	*B. discolor*	DQ912415	EF134549
EC	Kleinemonde	33°32′33.7″ S	27°01′56.7″ E	MHV 205	*C. monilifera*	DQ912416	EF134520
EC	Cannon Rocks	33°44′53.9″ S	26°32′03.2″ E	MHV 021	*B. discolor*/ *C. monilifera*	DQ912418	EF134521
EC	Cannon Rocks	33°45′07.1″ S	26°31′54.5″ E	MHV 391	*B. discolor*	DQ912417	EF134530
EC	Sundays River	33°42′54.7″ S	25°47′12.7″ E	MHV 390	*C. monilifera*	DQ912419	EF134529
EC	Cape Receiffe	33°59′51.2″ S	25°40′55.9″ E	MHV 389	*C. monilifera*	DQ912420	EF134527
EC	Schoenmakerskop	34°02′30.1″ S	25°33′01.0″ E	MHV 267	*C. monilifera*	DQ912421	EF134531
EC	Sea View	34°01′06.1″ S	25°22′11.2″ E	MHV 268	*C. monilifera*	DQ912422	EF134535
CC	Jeffreys Bay	34°00′59.6″ S	24°54′48.3″ E	MHV 269	*C. monilifera*	DQ912423	EF134534
CC	Cape St Francis	34°11′09.3″ S	24°49′16.6″ E	MHV 388	*C. monilifera*	DQ912424	EF134533
CC	Prince Albert Pass	33°51′53.1″ S	23°09′10.2″ E	MHV 318	*M. muricata*	DQ912427	EF134532
CC	Nature's Valley	33°58′23.2″ S	23°32′53.3″ E	MHV 376	*C. monilifera*	DQ912425	EF134541
CC	nr Plettenberg Bay	33°59′31.6″ S	23°26′10.5″ E	MHV 375	*M. muricata*	DQ912426	EF134540
CC	nr Mosselbay	34°06′03.6″ S	22°07′02.6″ E	MHV 374	*C. monilifera*	DQ912429	EF134526
CC	Knysna	34°01′56.8″ S	22°59′20.6″ E	MHV 271	*T. camphoratus*	DQ912428	EF134542
CC	nr Albertinia	34°10′19.1″ S	21°21′37.1″ E	MHV 373	*C. monilifera*	DQ912431	EF134539
CC	Witsand	34°23′22.9″ S	20°52′02.2″ E	MHV 272	*C. monilifera*	DQ912435	EF134545
CC	Arniston	34°39′38.1″ S	20°13′13.5″ E	MHV 274	*C. monilifera*/ *M. muricata*	DQ912434	EF134537
CC	Bredasdorp	34°37′04.5″ S	20°10′25.4″ E	MHV 273	*C. monilifera*	DQ912430	EF134538
CC	Struisbaai	34°46′44.9″ S	20°01′53.3″ E	MHV 275	*C. monilifera*/ *M. muricata*	DQ912433	EF134543
CC	nr Struisbaai	34°45′46.8″ S	20°01′47.0″ E	MHV 276	*C. monilifera*/ *M. muricata*	DQ912432	EF134544
WC	Die Dam	34°45′00.6″ S	19°40′40.3″ E	MHV 277	*C. monilifera*/ *M. muricata*	DQ912437	EF134546
WC	nr Gansbaai	34°35′30.0″ S	19°21′08.4″ E	MHV 278	*C. monilifera*	DQ912436	EF134547
WC	Hermanus	34°25′01.0″ S	19°12′57.9″ E	MHV 279	*C. monilifera*	DQ912438	EF134548
WC	Pringle Bay	34°17′47.8″ S	18°49′20.2″ E	MHV 310	*M. muricata*	DQ912439	EF134528
WC	nr Melkboschstrand	33°46′39.6″ S	18°28′04.3″ E	MHV 371	*C. monilifera*	DQ912443	EF134525
WC	Cape Point	34°14′51.8″ S	18°28′29.0″ E	MHV 372	*T. camphoratus*	DQ912440	EF134536
WC	nr Melkboschstrand	33°45′24.4″ S	18°27′22.8″ E	MHV 370	*M. muricata*	DQ912444	EF134522
WC	Koeberg	33°38′26.0″ S	18°26′42.1″ E	MHV 369	*C. monilifera*	DQ912445	EF134524
WC	Fish Hoek	34°07′53.3″ S	18°26′17.0″ E	MHV 368	*C. monilifera*	DQ912441	EF134523
WC	Scarborough	34°12′06.7″ S	18°22′55.8″ E	MHV 367	*C. monilifera*	DQ912442	EF134519
EM	nr Uniondale	33°42′21.8″ S	23°09′49.4″ E	MHV 152	*C. graminea*	DQ912477	EF134560
EM	Xagodi San	33°44′22.9″ S	23°46′30.4″ E	MHV 382	*M. muricata*	DQ912470	EF134563
EM	Joubertina	33°49′37.6″ S	23°52′36.9″ E	MHV 381	*M. muricata*	DQ912475	EF134598
EM	nr Joubertina	33°53′34.3″ S	24°06′08.5″ E	MHV 383	*M. muricata*	DQ912474	EF134557
EM	Kareedow	33°56′33.0″ S	24°16′04.4″ E	MHV 384	*M. muricata*	DQ912473	EF134564
EM	nr Kareedow	33°58′58.8″ S	24°30′39.9″ E	MHV 385	*M. muricata*	DQ912476	EF134565
EM	nr Humansdorp	33°59′41.7″ S	24°43′19.5″ E	MHV 377	*M. muricata*	DQ912478	EF134553
EM	nr Humansdorp	33°59′42.4″ S	24°43′09.4″ E	MHV 270	*M. muricata*	DQ912469	EF134570
EM	Jeffreys Bay	34°00′49.7″ S	24°53′04.3″ E	MHV 392	*M. muricata*	DQ912472	EF134566
EM	nr Humansdorp	34°03′14.0″ S	24°29′33.5″ E	MHV 393	*M. muricata*	DQ912471	EF134567
CM	nr Barrydale	34°01′26.9″ S	20°35′46.4″ E	MHV 317	*C. graminea*	DQ912466	EF134579
CM	Houwhoek Pass	34°13′37.6″ S	19°11′15.3″ E	MHV 379	*M. muricata*	DQ912468	EF134554
CM	Houwhoek Pass	34°13′44.0″ S	19°11′15.2″ E	MHV 288	*C. ruscifolia*	DQ912465	EF134572
CM	Theewaterskloof Dam	34°03′23.0″ S	19°08′44.3″ E	MHV 291	*C. graminea*	DQ912467	EF134587
WM	nr Worcester	33°33′20.0″ S	19°30′55.0″ E	MHV 312	*C. graminea*	DQ912451	EF134578
WM	Bainskloof Pass	33°33′07.8″ S	19°09′31.3″ E	MHV 303	*M. muricata*	DQ912462	EF134573
WM	nr Bainskloof Pass	33°30′49.4″ S	19°11′29.2″ E	MHV 306	*C. ruscifolia*	DQ912457	EF134574
WM	nr Worcester	33°36′10.6″ S	19°20′12.3″ E	MHV 307	*C. ruscifolia*	DQ912448	EF134575
WM	Dutoitskloof Pass	33°42′15.2″ S	19°14′25.3″ E	MHV 309	*C. ruscifolia*	DQ912454	EF134576
WM	Dutoitskloof Pass	33°44′42.8″ S	19°03′04.6″ E	MHV 311	*C. ruscifolia*	DQ912461	EF134577

Table 1 *Continued*

Clade	Locality	Latitude	Longitude	Collection #	Host plants	GenBank no. (COI)	GenBank no. (16S)
WM	Franschhoek	33°52′42.7″S	19°01′48.6″E	MHV 296	*M. muricata*	DQ912458	EF134591
WM	Boschendal	33°53′03.3″S	18°58′02.6″E	MHV 299	*C. polygonifolia*	DQ912455	EF134594
WM	Franschhoek Pass	33°55′17.8″S	19°08′17.9″E	MHV 294	*Eucalyptus* sp.	DQ912456	EF134589
WM	Jonkershoek	33°58′07.0″S	18°56′04.1″E	MHV 300	*C. ruscifolia*	DQ912453	EF134595
WM	Stellenbosch	33°59′01.2″S	18°50′10.9″E	MHV 281	*C. ruscifolia*	DQ912449	EF134581
WM	Theewaterskloof Dam	34°00′51.7″S	19°12′18.5″E	MHV 292	*M. muricata*	DQ912460	EF134588
WM	nr Elgin	34°11′15.0″S	19°05′39.8″E	MHV 287	*M. muricata*	DQ912450	EF134584
WM	nr Grabow	34°07′09.8″S	19°03′03.3″E	MHV 290	*C. ruscifolia*	DQ912452	EF134586
WM	Sir Lowry's Pass	34°08′04.1″S	18°55′31.1″E	MHV 394	*C. ruscifolia*	DQ912446	EF134558
WM	Sir Lowry's Pass	34°08′04.2″S	18°55′30.6″E	MHV 283	*C. ruscifolia*	DQ912464	EF134600
WM	Sir Lowry's Pass	34°08′59.7″S	18°56′04.4″E	MHV 284	*M. muricata*	DQ912463	EF134583
WM	Grabow	34°09′42.0″S	19°00′37.9″E	MHV 380	*Platanus* sp.	DQ912447	EF134556
WM	Grabow	34°09′42.2″S	19°00′37.9″E	MHV 286	*C. ruscifolia*/ *M. muricata*	DQ912459	EF134571

Table 2 Locality, host plant, voucher number and GenBank accession no. of *Platypleura stridula*, *Platypleura* sp. 10 and outgroup samples

Clade	Locality	Latitude	Longitude	Collection #	Host plants	GenBank no. (COI)	GenBank no. (16S)
P. stridula	Franschhoek	33°54′50.2″S	19°07′25.3″E	MHV 180	unidentified	DQ912482	EF134561
P. stridula	Dwarsrivier Farm	32°13′14.4″S	18°59′19.8″E	MHV 024	*Salix babylonica*	DQ912488	EF134555
P. stridula	Citrusdal	32°35′48.0″S	19°00′30.0″E	MHV 001	unidentified	DQ912487	EF134568
P. stridula	Algeria	32°43′00.0″S	19°03′00.0″E	MHV 025	unidentified	DQ912489	EF134559
P. stridula	Bainskloof Pass	33°33′21.4″S	19°08′59.1″E	MHV 302	*Salix mucronata*	DQ912490	EF134596
P. stridula	nr Worcester	33°36′10.6″S	19°20′12.4″E	MHV 341	*C. graminea*	DQ912492	EF134597
P. stridula	nr Paarl	33°47′35.2″S	18°57′09.1″E	MHV 297	*Eucalyptus* sp.	DQ912483	EF134592
P. stridula	nr Pniel	33°52′04.3″S	18°58′36.0″E	MHV 298	*Platanus x hispanica*	DQ912485	EF134593
P. stridula	Franscheshoek	33°53′00.4″S	19°04′15.7″E	MHV 295	*Platanus x hispanica*	DQ912486	EF134590
P. stridula	Cloetiesville	33°54′18.0″S	18°51′32.3″E	MHV 280	*Leucas* sp.	DQ912480	EF134580
P. stridula	Cloetiesville	33°54′09.0″S	18°51′25.8″E	MHV 029	unidentified	DQ912481	EF134569
P. stridula	Onderpapagaaiberg	33°56′27.0″S	18°49′11.0″E	MHV 412	unidentified	DQ912479	EF134599
P. stridula	Stellenbosch	34°00′10.4″S	18°49′28.3″E	MHV 282	*C. ruscifolia*	DQ912484	EF134582
P. stridula	Caledon	34°13′41.9″S	19°25′48.3″E	MHV 289	*S. babylonica*	DQ912493	EF134585
P. stridula	nr Elgin	34°14′05.3″S	19°11′30.8″E	MHV 378	*Eucalyptus* sp.	DQ912491	EF134562
P. sp. 10	Van Rhynsdorp Pass	31°23′00.0″S	19°01′00.0″E	MHV 010	unidentified	EF134605	EF134551
P. sp. 4	Ghwarriepoort River	33°23′18.0″S	23°23′15.0″E	MHV 323	*Acacia karroo*	EF134604	EF134552
P. hirtipennis	Brentwood Farm	33°29′37.0″S	26°09′15.0″E	MHV 195	unidentified	EF134602	EF134601
P. walhbergi	East London	32°57′27.0″S	27°56′03.0″E	MHV 365	*Berkheya heterophylla*	EF134603	EF134550

Total genomic DNA was extracted from the muscles following the Chelex® 100 protocol (Walsh *et al*. 1991). Small pieces of tissue (*c*. 2 mm^3) were sliced finely using a sterile scalpel blade and placed in 5% Chelex extraction buffer (150μL of 20% Chelex 100 solution, 450μL TE buffer (10 mM Tris, 1 mM EDTA)) and incubated at 60°C for two hours, and then at 100°C for 15 min in a heating block. Samples were then centrifuged at 13 000 r.p.m. for 1 minute and the supernatant removed for subsequent use in Polymerase chain reaction (PCR) amplifications.
A 525-base-pair region of the mitochondrial large subunit ribosomal 16S RNA gene was amplified and sequenced using the primers 16S A and 16S B (Palumbi *et al*. 1991), and an 816-base-pair portion of the 3′ end of the Cytochrome Oxidase I (COI) gene was amplified using the primers C1-J-1718 (Simon *et al*. 1994) and C1-N-2568

(Brady *et al*. 2000). The difficulty of amplifying some taxa resulted in the development of an internal primer: (5′-GTATCATGYAARACAATATCAAT-3′), being designed to replace C1-N-2568 for PCR. PCR amplifications were confirmed by electrophoresis of 5 μL PCR product and 5 μL tracking dye in a 1% agarose gel, stained with ethidium bromide and visualized using a UV trans-illuminator.
PCR products were purified using the Wizard® SV quick purification kit (Promega Corp.) and sequenced in both directions using the flanking primers for each region and the two internal primers C1-N-2191 and C1-J-2183 (Simon *et al*. 1994) for the COI region. The sequencing reaction was carried out using the ABI Big Dye Sequencing kit version 3.1, according to the manufacturer's instructions. Sequence trace files were generated using an ABI 3100 genetic analyzer sited at Rhodes University. Trace files were checked and edited using sequencher version 3.01 (Gene Codes Corporation). The sequence data were imported into MCCLADE version 4.06 (Maddison & Maddison 2000) and aligned manually.

Phylogenetic analysis

To ascertain whether the data from each gene region could be combined into a single data set, the Incongruence Length Difference (partition homogeneity) test (Farris *et al*. 1994) was conducted in PAUP* version 4.0b10 (Swofford 2002) with invariant characters removed (Cunningham 1997) because the number of variable characters differed between the 16S and COI data sets.
Three different methods of phylogenetic analysis were performed: Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI). An unweighted MP analysis was conducted using PAUP* as follows: 100 random addition replicates were conducted keeping a single shortest tree for each replicate (TKEEP = 1). All trees retained in memory from this process were then swapped to completion using a HEURISTIC search with TBR branch swapping. Confidence in each node was assessed with 1000 FULL HEURISTIC bootstrap replicates.
The most appropriate model of sequence evolution was selected using the AIC test (Akaike 1974) as implemented in MODELTEST version 3.7 (Posada & Crandall 1998). ML analysis was conducted under this model in PAUP*.
The BI analysis was conducted using MRBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001) under the best fitting model as selected by the AIC test in MRMODELTEST version 2.2 (Nylander 2004). The MRBAYES analysis comprised four independent runs of 2 000 000 generations using random starting trees with four chains (one cold, three hot), sampling every 100 generations. Plots of likelihood scores, tree length and average standard deviation (SD) of split frequencies against number of generations showed that the analysis reached stationarity well within the first 10% of trees generated. Thus the first 10% of trees generated were discarded, ensuring that only trees generated at stationarity were used to calculate the BI posterior probabilities. The mean intra- and net interclade percent sequence divergence was calculated in mega version 3.1 (Kumar *et al*. 2004) using the Kimura 2-parameter model (Kimura 1980).

Molecular dating

Although the application of a global molecular clock is problematic (Buckley *et al*. 2001; Heads 2005), the COI region has been shown to be the most reliable gene when enforcing a molecular clock for insects because it possesses the most consistent rates of sequence evolution between lineages (Gaunt & Miles 2002). The data were tested for equal substitution rates across the tree using RRTREE version 1.1 (Robinson-Rechavi & Huchon 2000) and the topology of the BI analysis. Furthermore, they were tested for the applicability of a molecular clock using two separate ML analyses; first with and then without the molecular clock enforced. The likelihood ratio test (LRT) was then used in combination with a Chi-squared test to assess whether or not the molecular clock significantly influenced the ML analysis (Felsenstein 1981).
Without fossil evidence or an adequate geological vicariance event to calibrate the molecular clock, we resorted to a bounded estimate of the timing of divergence using both the 16S and COI gene regions independently. The mean rate of sequence evolution was obtained for each region from previously published rates, using hemimetabolous insect taxa calibrated to within the last 10 million years. The mean rates used in this study were COI = 2.25% per MY (Brower 1994; Buckley *et al*. 2001) and 16S = 1.4% per MY (Brower 1994). The time to the most recent common ancestor (MRCA) between each clade was then estimated under the model parameters

highlighted in MRMODELTEST using a Bayesian approach in beast 1.4 (Drummond & Rambaut 2003) with 5 000 000 steps, following a discarded burn-in of 50 000 steps. Convergence to stationarity and the estimation of effective sample size (ESS) in each analysis was confirmed by inspection of the MCMC samples using TRACER 1.2 (Rambaut & Drummond 2003).

Acoustic analysis

Cicada calls were recorded at multiple localities for each major clade resolved by the phylogenetic analysis using a Sony MZ-NH1 Minidisc Recorder and Sony ECM-719 stereo microphone. Sonograms were produced for each call using RAVEN version 1.2 (Charif *et al*. 2004) with default settings. Both within- and between-clade variability in the frequency range and length of each call echeme were analysed with separate two-way anovas as implemented in STATISTICA version 7 (StatSoft Inc.); significant pairwise differences were analysed using Scheffe's test.

Morphological analysis

Samples from each clade were pinned as voucher specimens. The morphology of specimens in each clade within the group is very similar, and the most morphologically variable body part is the males' 10th abdominal segment, the urite of their genitalia. A preliminary analysis was conducted using measurements from both the forewing and the urite (Fig. 3A) of specimens from each clade except *Platypleura* sp. 10, for which too few specimens were available for meaningful analysis. Wing measurements were obtained at 6× magnification, using a WILD M5A stereo microscope (Leica, Switzerland) and eye-piece graticule. Urite measurements were obtained at 35× magnification using a Leica EZ4D stereo microscope (Leica, Switzerland) and the Leica Application Suite version 2.3 (Leica Microsystems, Switzerland) image analysis software. The data were log-transformed to linearize allometric covariation and analysed using a correlation matrixbased principle component analysis (PCA) in STATISTICA.

Results

Phylogenetic analysis

The final molecular data set consisted of 1341 base pairs, comprising 816 base pairs of COI data that aligned readily and 525 base pairs of 16S data that aligned readily with some gaps corresponding to insertion or deletion events. The ILD test indicated that the data sets were not significantly different ($P = 0.41$), so the data were combined. Of the 1341 base pairs, 258 characters were variable (19%); of these, 176 characters (13%) were parsimony-informative. All analyses resolved six well-supported clades within the *Platypleura stridula* complex, but the topological arrangement of some of the clades lacked support (Fig. 1).

Parsimony analysis yielded 32 most-parsimonious trees with a tree length of 340 steps (CI = 0.653; RI = 0.951). The TrN + I + G model was selected as the best model of sequence evolution for the combined data set using the Akaike information criteria (Akaike 1974) in modeltest. ML analysis under this model yielded one most likely tree (ln L = −4076.2019 & ln L = −4111.9915 clock enforced).

Bayesian analysis under the most similar model implemented in MRBAYES 3.1.2 (GTR + I + G) also yielded one tree (Fig. 1), on which the MP bootstrap and the BI posterior probability values for each of the nodes are shown. The clades found in all analyses included a monophyletic *P. stridula* clade (sister to *Platypleura* sp. 10) and four other clades, comprising specimens all identified as *P. capensis* following Villet (1989). For this study, the clades were labelled as the *P. stridula* clade (P.s), the Coastal clade (C) and the East (EM), West (WM) and Central Montane (CM) clades (Fig. 1). The three Montane clades lacked wellsupported substructure, but closer inspection of the BI tree showed that the Coastal clade can be further subdivided into well-supported Eastern [EC], Central [CC] and Western [WC] lineages and the *P. stridula* clade can be further subdivided into well-supported Northern [N], Southern [S] and Eastern [E] lineages (Fig. 1).

The Kimura 2-parameter distance of the ingroup samples showed no overlap between the within-clade mean (COI: 0.1–1.4%; 16S: 0.0–0.5%) and the net between-clade (COI: 2.3–6.3%; 16S: 1.4–4.6%) divergence.

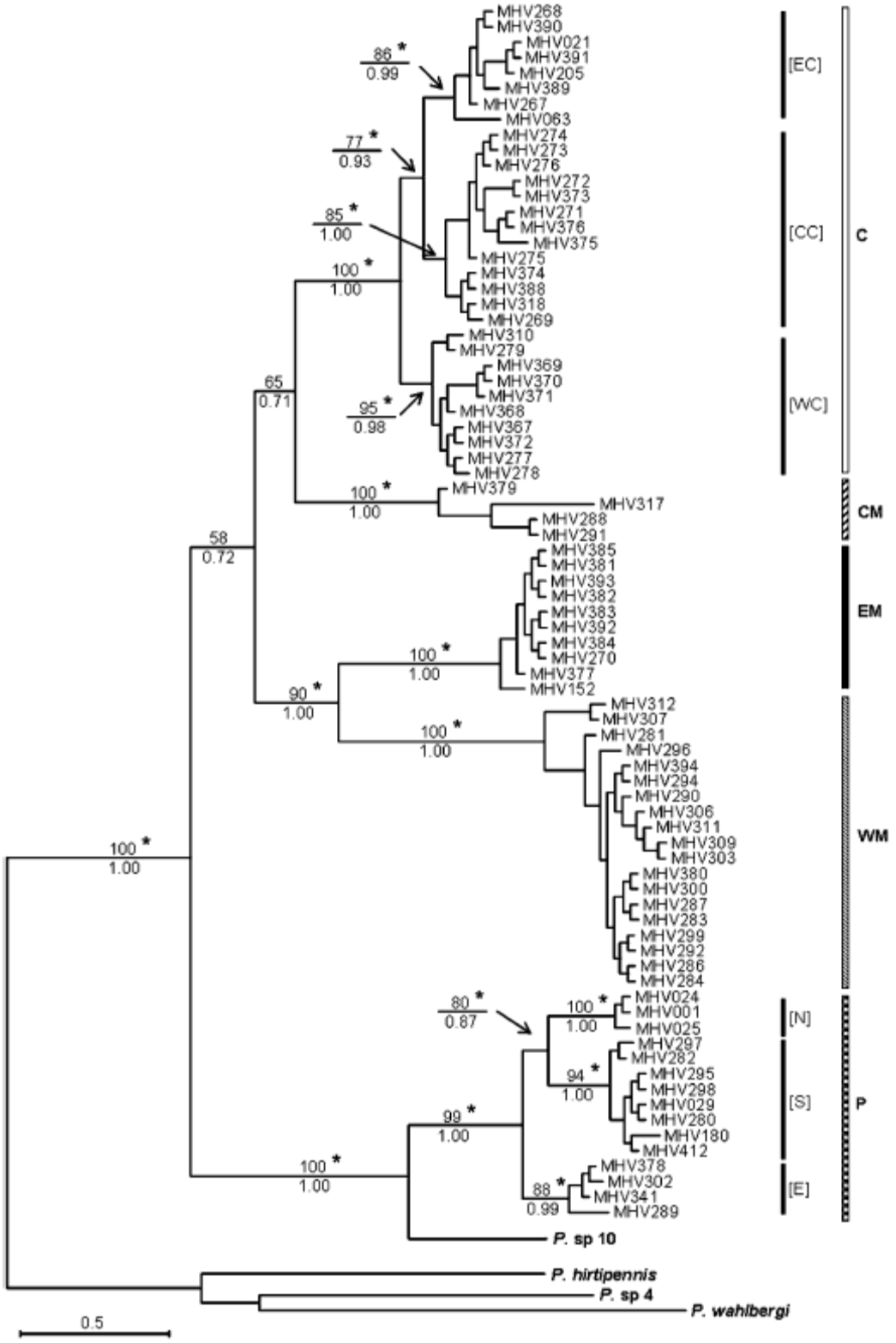

Fig. 1 Majority rule Bayesian Inference phylogeny highlighting five major clades: Platypleura stridula (P), Coastal (C) and the Western (WM), Central (CM) and Eastern (EM) Montane clades in addition to three subclades within P. stridula: Northern [N], Southern [S] and Eastern [E] and Coastal: Eastern [EC], Central [CC] and Western [WC] clades. Statistical support generated in the analyses for each node is shown as MP bootstrap above and BI posterior probability below each line, each node present in the ML tree is indicated with (*).

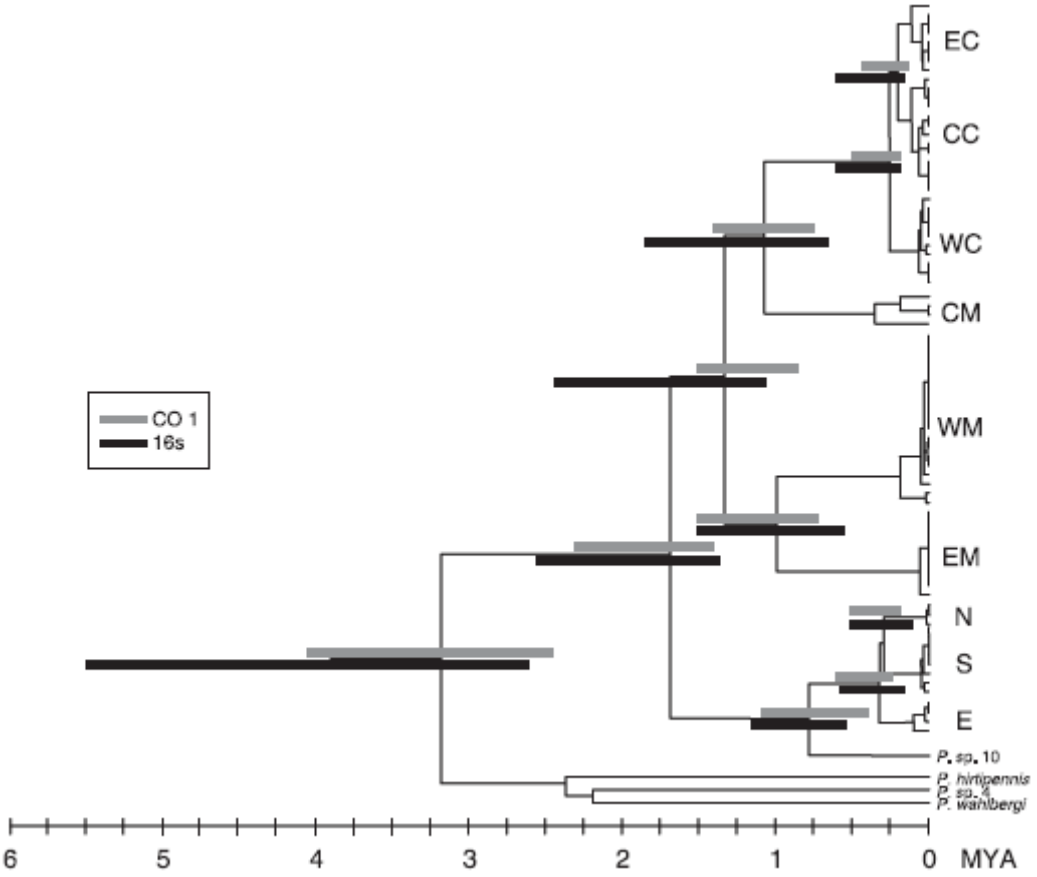

Fig. 2 Clock-enforced Maximum-Likelihood tree showing the estimated time to the most recent common ancestor (MRCA) between each clade. Bars denote the upper and lower bounds of the 95% HPD interval as estimated in BEAST, using COI (grey) and 16S (black) regions independently.

Molecular dating

Both the LRT ($P = 0.76$) and the relative rate tests ($P = 0.19$) showed that the rate of sequence evolution did not differ significantly between major clades. Dating estimates on the sequence data indicated that radiation within the in-group occurred within the last 2.4–5.5 million years. The vicariance of the three *P. stridula* lineages and the three Coastal lineages is estimated to have occurred more recently, within the last 0.11–0.56 and 0.13–0.65 MY, Respectively (Fig. 2).

Acoustic analysis

Sonograms of the calls recorded at one site for each of the major clades showed that the emphasized frequencies differ between clades. Lower frequencies were utilized by *P. stridula* (6–8 kHz), *Platypleura* sp. 10 (4–8 kHz) and the Coastal clade (6–13 kHz), while the Montane clades emphasized a higher frequency range (9–14 kHz). In addition, the Coastal and Montane clades and *Platypleura* sp. 10 show frequency modulation, a trait absent in *P. stridula*. Anova of the Coastal and three Montane clade-call echemes showed high within-clade variability and no significant difference ($P > 0.05$) in the length of the call echeme between clades.

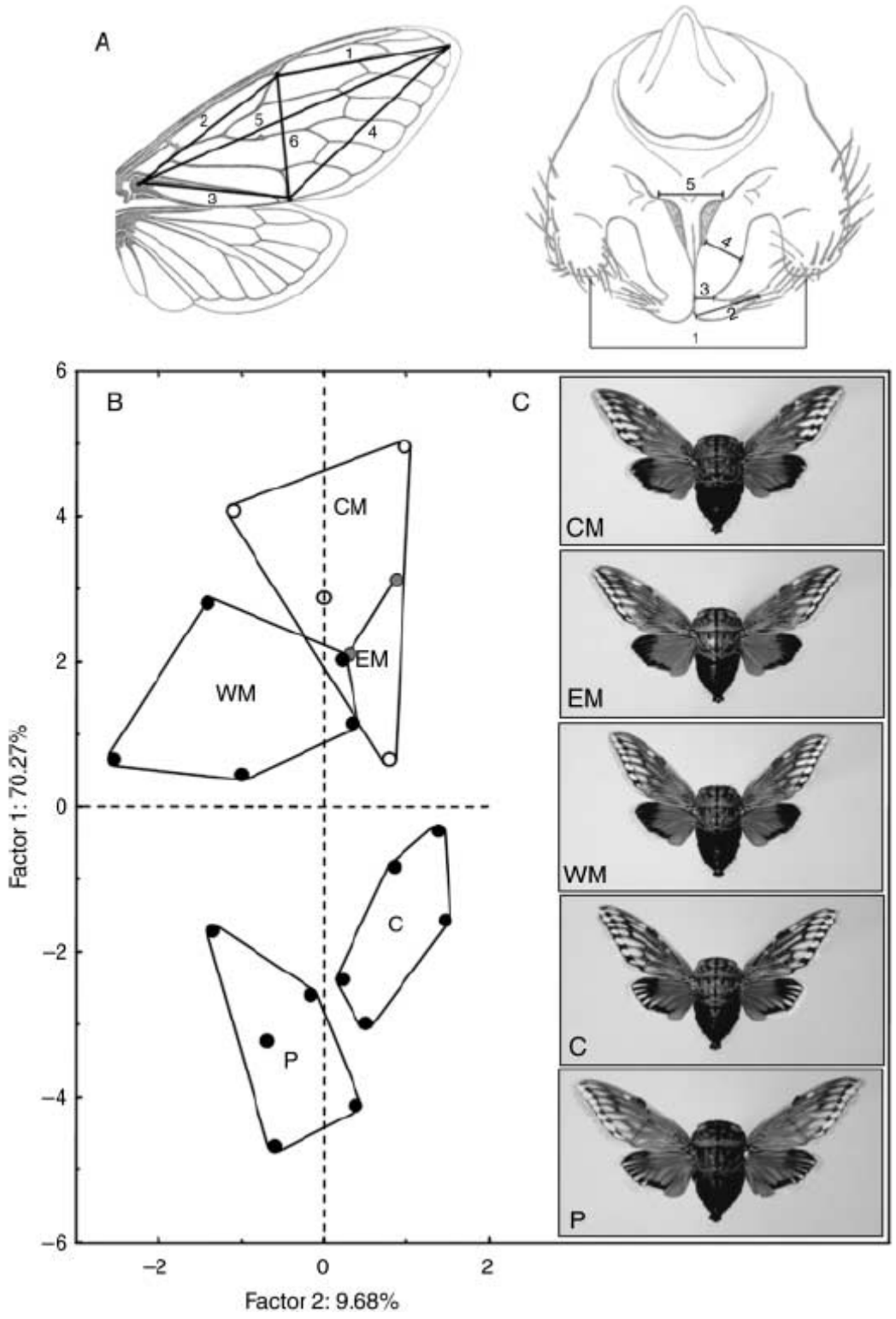

Fig. 3 (A) Diagram of right wings and urite showing measurements used in the Principal Component Analysis (PCA), (B) plot of the first two PCs of the PCA, with some horizontal exaggeration of the second component; convex hulls enclose samples of each of the major clades, (C) dorsal view of a specimen characteristic of each clade.

Morphological analysis

The first two PCs of the PCA of wing and urite measurements (Fig. 3B) described 80% of the variation in the samples (Table 3), while the remaining factors did not significantly describe variation between the specimens. The plot of the first two factors showed three distinct groupings: *P. stridula*, Coastal and the combined Montane clade specimens. Specimens of the three Montane clades were distinctly smaller than those of the other clades, which were slightly different in size. All clades overlapped on the second axis and are not readily distinguishable.

Discussion

Taxonomy

The recovery of six lineages from samples of what were previously two nominal species was surprising. Should each clade receive species status? Assigning species status to organisms occurring in allopatry has proved to be more difficult than with organisms occurring in sympatry, especially when the taxa concerned are morphologically cryptic (Daniels et al. 2003). In particular the Central and Western Montane clades occur in such close proximity (within 10 km in two pairs of samples) that they are apparently parapatric, yet this has not been rigorously established. As a result we have attempted to take a more holistically biological approach as emphasized by Sites & Marshall (2004), utilizing genetic, morphological and courtship data when approaching this question. The mtDNA data distinguish six clades with high levels of statistical support, suggesting the applicability of a species concept akin to the Phylogenetic Species Concept (Mishler & Donoghue 1982; Cracraft 1983; Zink & McKitrick 1995). The clades show levels of sequence divergence that warrant species rank using previously cited thresholds of DNA variability (Hebert *et al*. 2003; Kartavtsev & Lee 2006; Smith *et al*. 2006). Furthermore, the comparable divergence in the mtDNA COI data between these clades and between recognized species within the genus (data from Villet *et al*. 2004) indicate that all six clades warrant species status. The lack of overlap between the COI intraclade (< 1.4%) and interclade (> 2.3%) divergence may argue the usefulness of this region in species delineation (Kartavtsev & Lee 2006). That said, there are hazards in interpreting genetic distance as a criterion for species delineation (Ferguson 2002; Meyer & Paulay 2005; Brower 2006), and we favour the more holistic and biological approach of species delineation involving morphological and acoustic data.

The overall size of the individuals within the complex (Fig. 3) enables the identification of three distinct morphospecies represented by the *Platypleura stridula*, Coastal and combined Montane clades. *Platypleura* sp. 10 is similar in size to *P. stridula*. Morphology alone does not separate the three Montane clades and has proven to be a generally problematic basis for a species concept within the group. This is expected given the crypsis and sexual signaling channels exhibited in the group (Villet 1989) and the proposed recent diversification of the Montane taxa. Platypleurine cicadas show only rudimentary courtship (Villet *et al*. 2003), so that most of the interaction between the sexes occurs through long-range acoustic signals (Villet 1992), which are therefore the major determinant of gene flow through mating (cf. Paterson 1985; Villet 1995; Quartau *et al*. 2000). In this regard the acoustic data indicate at least four species: *P. stridula*, *Platypleura* sp. 10, Coastal and combined Montane, based on frequency modulation and the peak frequency of the call. No difference in length of call echemes between clades was observed, due to within-clade variability in echeme duration. Although some cicadas have been shown to exhibit fine-scale pitch discrimination (Fonseca *et al*. 2000), the present acoustic data are insufficiently divergent between the three Montane clades to interpret each of them as distinct biological species under the 'Specific Mate Recognition System' species concept (Paterson 1985; Villet 1995). However, these data are preliminary and suggest a worthwhile avenue for further investigation. Samples of the Coastal and Eastern Montane clades were collected within 3 km of one another at Jeffreys Bay (Table 1) and probably occur within 1 km of one another at the southern extreme of the distribution of the Western Montane clade, so that the Coastal and Montane taxa are practically sympatric in places. This provides strong support to the morphological and acoustic data for interpreting the Coastal clade as a distinct species from the Montane clades. As a result of a lack of similar evidence regarding the taxa within the Montane group, we suggest that at present they should be considered as three distinct evolutionary significant units (Ryder 1986; Moritz 1994) for conservation and management purposes, pending further evidence (both acoustic and morphological) that these three clades warrant species status.

Preliminary comparisons by one of us (MHV) of photographs of the putative type specimens of *P. stridula* and *P. capensis*, held in the Uppsala University Museum collection, confirm the identity of *P. stridula*. However, the identity of the putative type specimen of *P. capensis* is ambiguous, requiring physical scrutiny. The nomenclature of the Coastal and three Montane clades with regard to *P. capensis*, *Cicada nigrolinea* and *C. catenata* will be addressed at a later date once all types have been scrutinized.

Table 3 Eigenvectors and Eigenvalues of a Principal Component Analysis of the body measurements defined in Fig. 3(A). Factor 1 may be interpreted as an expression of overall body size. Factor 2 summarizes variation in two of the processes of the urite. The species are differentiated fairly well by body size, and poorly by genitalia

		Principal components			
Variable		1	2	3	4
Wing	1	**–0.353**	0.041	0.135	–0.084
	2	**–0.348**	0.090	0.084	–0.047
	3	**–0.352**	0.042	0.174	–0.048
	4	**–0.346**	0.044	0.125	0.022
	5	**–0.344**	0.149	0.078	–0.101
	6	**–0.351**	0.101	0.096	–0.101
Urite	1	**–0.273**	–0.121	–0.212	–0.602
	2	–0.103	**–0.794**	0.520	0.206
	3	**–0.275**	0.079	–0.117	0.382
	4	**–0.270**	0.104	–0.290	0.641
	5	–0.181	**–0.538**	–0.706	–0.050
Eigenvalue		7.729	1.064	0.772	0.619
Cumulative variance (%)		70.27	79.95	86.97	92.60

Plant association

The host-plant data (Tables 1 and 2) suggest that the species within the complex show a wide range of plant specificity with strict plant association in the Montane clades, oligophagy in the Coastal clade and polyphagy in *P. stridula*. *Platypleura* sp. 10 is apparently associated with *Cliffortia* plants (MHV, unpublished), but current samples are not definitive. These differences are most likely a result of the plant associations being mediated via long-range visual cues, like overall plant structure (tree vs. shrub) and gross leaf morphology (broad-leaved vs. fine-leaved), rather than close-range chemical cues. Although the original host plant of *P. stridula* was most likely the Indigenous *Salix mucronata* Thunberg (MHV, unpublished), it can now be found on many large trees, most of which are recently introduced exotics (Table 2), lending support to the hypothesis of long range visual mediation of plant associations within this species. The large taxonomic discrepancy in the plant associations of the taxa within this group indicates shifts in plant associations as a mechanism for speciation within this group, based on the premise that recently diverged sister species are most likely to differ most in traits linked to speciation (Barraclough 2006). This result is not surprising, as the majority of speciation events in phytophagous insects are accompanied by host plant shifts (Futuyma 1991).

Biogeography

Although preliminary, the dating analyses are plausible first estimates of the date of divergence within this group, highlighting initial diversification at the Pliocene–Pleistocene boundary. The common association of *Platypleura* sp. 10 and the Montane clades with *Cliffortia* plants (MHV, unpublished) suggests that they represent models for

the ancestral taxon of the species complex. This ancestor was therefore probably also associated with montane habitats, implying that vicariance of such habitats into northern and southern blocks was an important initial step in the diversification of the group, but the mechanism is not obvious. Recent evidence (Weaver *et al*. 1998; Meadows & Baxter 1999) implies only a small impact of the last glacial maximum (a recent but severe glaciation) on the temperatures of the southwestern Cape, probably because it is a winter-rainfall region, so that climate is less likely to be a consideration. A more likely scenario involves events consequent on neotectonic uplift at about 2 Ma, driving initial diversification in this complex at about 1.8 Ma (Fig. 2). Subsequent diversification within both the northern and southern blocks produced all of the major extant clades at about 1.2–0.8 Ma (Fig. 2).

In all analyses, little support was generated for any particular topology of relationships between the Coastal and Montane clades. This result may be interpreted in one of two ways. Firstly, rapid evolution of mtDNA can reduce the resolution of deeper branches (Avise 2001). This is unlikely here because there is no evidence of saturation in either gene. Another interpretation is that cladogensis occurred either simultaneously or in rapid succession (Hoelzer & Melnick 1994), resulting in a polychotomy where practically no opportunity existed for character-state changes to occur, or in which there is no clade structure to resolve (Page & Phelps 1994). However, it has been argued that explanations involving simultaneous differentiation should be considered only as a last resort (DeSalle *et al*. 1994). That said, situations abound where simultaneous or near-simultaneous isolation may have occurred in the CFR, including rapid changes in sea levels (generally in step with ice ages) throughout the Pliocene, a prominent factor in theories of the origins of the subtropical thicket vegetation (Cowling *et al*. 2005) and the rapid diversification of the Cape flora (Richardson *et al*. 2001; Linder 2003). Additionally, neotectonic crustal uplift in the Pliocene at about 5 Ma and 2 Ma (Artyushkov & Hofmann 1998) may have been a great stimulus for the diversification of plant lineages (Cowling & Proches 2005) by altering altitudes and accentuating a rain shadow in the western parts of southern Africa. The concomitant increase in erosion (Partridge & Maud 1987) also fragmented the substantially older Cape fold mountains with which the Montane clades are associated. Recent studies have shown similar disjunctions in both invertebrate and vertebrate taxa in the region, highlighting the complex topography of the region (Stevens & Picker 1999) and rapid sea level changes in the late Pliocene (Hendey 1983) as the most plausible explanations (Daniels *et al*. 2001; Wishart & Hughes 2001; Gouws *et al*. 2004).

What seems clear is that *P. stridula* and the Coastal clade both arose and developed new plant and habitat associations during this period. *Platypleura stridula* became associated with trees in drainage lines in areas west of the distribution of *Platypleura* sp. 10, while the Coastal clade moved to broad-leafed woody daisies generally growing within 200 m of the sea south of the distributions of the Montane clades. The coincidence of these changes may be fortuitous since, although they may be explained by altered plant-recognition cues, no common environmental cause is obvious.

The three distinct lineages of *P. stridula* originated almost simultaneously within the late Pleistocene. They show a similar geographical pattern to the tentative distribution of the species of 'heelwalkers' (Insecta: Mantophasmatodea) within the region (Klass *et al*. 2003). The *P. stridula* clade as a whole shows relatively large genetic distances between lineages that are each contained within a major watershed within the western Cape (Fig. 4A), suggesting that the mountains within this region provide significant barriers to gene flow in this species. Although catchments have been found to define the distributions of invertebrates with aquatic stages within the region (Wishart & Hughes 2001) it is unusual that *P. stridula*, which lacks an aquatic stage, is similarly circumscribed. In this case, habitat philopatry may interact with the mountain barriers to reduce gene flow between catchments. This may be a consequence of the fact that *P. stridula* is primarily associated with an endemic willow (*Salix mucronata* ssp. *hirsuta* (Thunberg) Immelman) that grows along river courses in the Olifants and Berg River catchments of the Western Cape region. River capture in the region (Hendey 1983) may allow the colonization of new catchments, but it is unlikely to produce three clades almost simultaneously. An additional evolutionary scenario is that the willows spread high into the headwaters in wetter periods, allowing the cicadas relatively easy migration between catchments, while range contraction of both willows and associated *P. stridula* populations in the cooler, drier Pleistocene glacial periods resulted in vicariance between catchments. Such a vicariance model, however, needs to be validated by palaeoclimatic data.

The Coastal clade shows three distinct lineages that originated at approximately the same time as those of *P. stridula* (Fig. 2) and conform to the supratidal biogeographical zones defined by Tinley (1985) (Fig. 3C). The boundary between the Western and the Central Coastal clades is centred on Cape Agulhas, whereas the boundary between the Central and Eastern Coastal clades is centred on the Gamtoos River valley (Fig. 4C). No obvious geographical barriers are present at clade boundaries that would impede recent migration within this species.

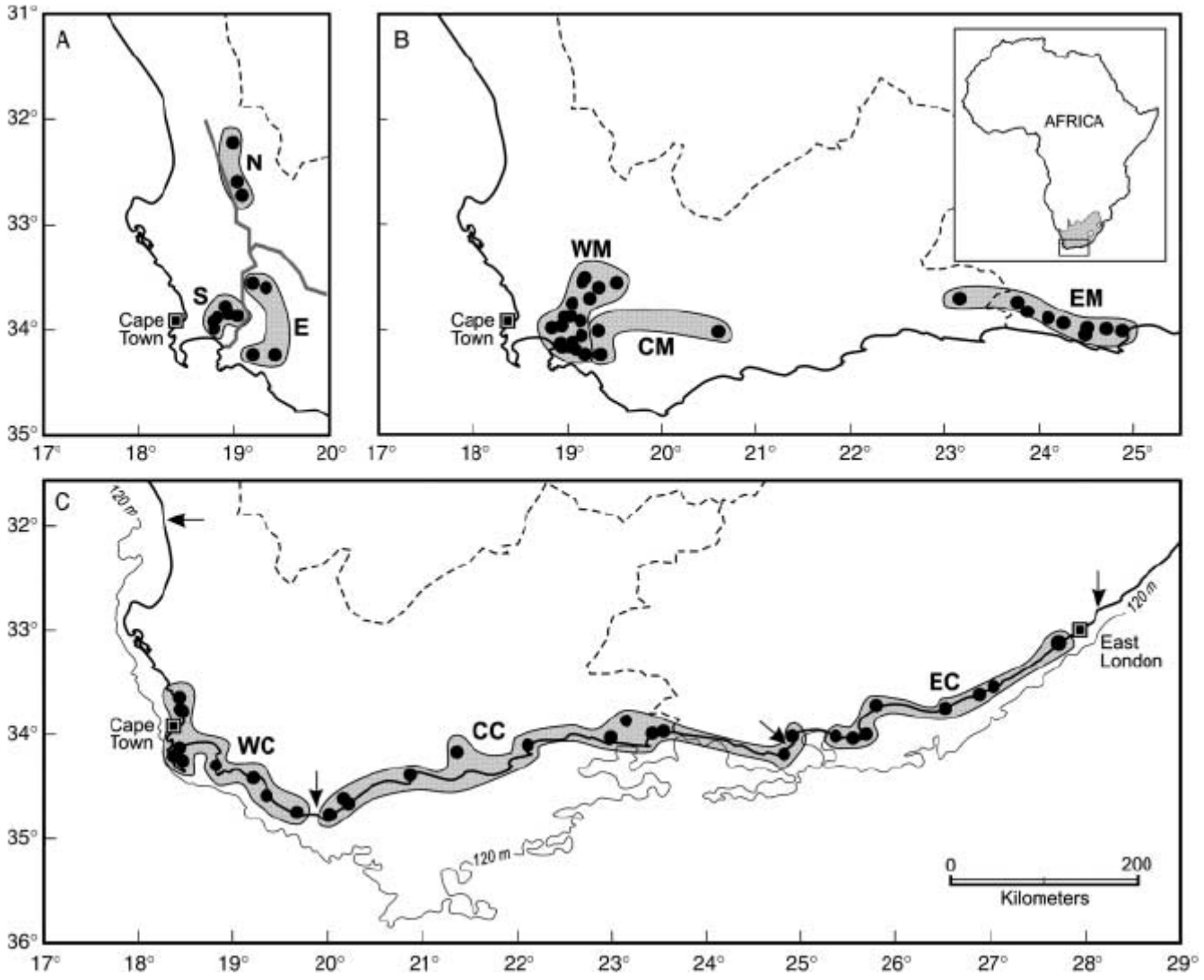

Fig. 4 Geographical distribution of clades found in the analyses (A) *Platypleura stridula* (B) combined Montane clades and (C) Coastal clade. Sample localities within each clade are shown as dots. Within the *P. stridula* clade Aa), solid lines indicate the position of the major watersheds. Within the Coastal clade (C), the solid line offshore indicates the −120 m isobath redrawn from the ETOPO 2′ Global Bathymetry Grid (National Geophysical Data Center 2001). Arrows show boundaries between the three supratidal zones within the region defined by Tinley (1985).

Preliminary dating of the split between the Western and Central Coastal clades indicates one or more vicariance events within the Pleistocene (662 000–197 000 years ago) as the most likely explanation. Within the Pleistocene, lower sea levels associated with glaciation (*c*. −120 m) would expose the majority of the marine Agulhas plain (Dingle & Rogers 1972), pushing the coastline over a degree of latitude further south of the present Cape Agulhas (Fig. 3C). The colder climate of this exposed region as a result of the glacial maximum, associated with the drop in sea levels, could have excluded the Coastal clade from the more southerly Agulhas region.

The apparent disjunction between the Central and Eastern Coastal clades mirrors that found in their associated plant *Chrysanthemoides monilifera* (L.) T. Norl. ssp. *Rotundata* (DC.) T. Norl. (Scott 1996) and could involve the Gamtoos River system, which might have acted as a physical barrier to gene flow by forming a large marine bay when inundated by higher sea levels than present. Preliminary dating between clades indicates the vicariance event leading to the separation of the two clades to have occurred in the recent Pleistocene (655 000–136 000 years ago). Indeed, marine transgressions that might result in division of the coastal population have been recorded in the

Pliocene (Siesser & Dingle 1981), but no inundations above 6 m have been recorded in the Pleistocene (Hendey 1983). A more likely explanation is the exposure of unsuitable habitat offshore of the present position of the Gamtoos River during Pleistocene glacial periods as a result of the close proximity of the 0 m and −120 m isobaths (Fig. 4C), forming steep shores when sea levels dropped. The secondary effects of climate changes, such as sea level change and the associated exposure of unsuitable habitat, seem the most likely causes of the modern pattern. Thus, although there is evidence of only limited local climate change in the southwestern Cape (Weaver *et al*. 1998; Meadows & Baxter 1999), the effects of glaciation elsewhere could still affect this buffered environment indirectly. In addition, because of peculiarities in the submerged topography of the Gamtoos valley and St Francis Bay region, changes in sea level cause very substantial changes in the length of the local coastline (Fig. 4C), as they do on the Agulhas plain. This would result in the alternating dispersion and compression of coastal populations, which have an essentially one-dimensional distribution. The effects of such processes on population genetics need to be explored further, since they potentially affect all species restricted to narrow coastal distributions.

This study has highlighted the effect of different mechanisms as drivers of cicada diversification in the CFR and highlights the importance of using multiple sources of data when differentiating cryptic sister taxa. The combination of phylogenetic and ecological perspectives provides the time frame and possible scenarios driving diversification and speciation in this group of CFR insects, which is most likely a result of the combination of Pliocene–Pleistocene uplift, climate change and associated diversification and radiation of the fynbos flora, as well as Pleistocene vicariance associated with the interaction of sea-level changes and local coastal topography.

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