The life-history and relationships of ‘Orthocladius’ pictipennis Freeman (Diptera: Chironomidae)

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ABSTRACT

The previously unknown larva and pupa of ‘Orthocladius’ pictipennis Freeman have been found, and associated by molecular means. Pharate pupae (males within pupae) allow the link to the described adult. We describe the larva and pupa, and provide short notes on the adult. The taxon is unrelated to Orthocladius – no members of this Holarctic genus are present in New Zealand – and therefore we provide a new generic name, Paulfreemania Cranston and Krosch gen. n, as well as a short discussion of relationships amongst austral Orthocladiinae.

INTRODUCTION

Studies of New Zealand Chironomidae (Diptera) inevitably reveal previously unreported diversity of the aquatic immature stages, especially amongst the small-sized and morphologically cryptic taxa in the subfamily Orthocladiinae. Perhaps less usual is the discovery of previously unknown life histories of taxa described when the adult stages were the sole subject of study. The seminal monographic treatment of the New Zealand adult midges was that of Freeman (1959) who synthesised previously disparate taxonomic accounts into a classification that came at the cusp of transition from adult-based to include the immature stages. In the past half century, the described diversity of the important subfamily Orthocladiinae has increased particularly through the studies of Boothroyd (e.g., Boothroyd 1994, 1999, 2004) and Cranston (Cranston 2007, 2009; Cranston & Saether 2010). Our increased understanding of the identity and postulated gondwanic relationships of certain of these taxa encouraged the second author to undertake phylogeographic and phylogenetic studies on austral members of the Orthocladiinae, including those found in running waters of the northern part of South Island, New Zealand. In the course of these studies, which involved screening large numbers of very similar-looking small larvae for a phylogeographic study (Krosch et al. in press), a cluster of specimens associated with no described taxon was encountered in several streams. This cluster could be associated with two pharate specimens (that is, well formed adult male within its pupa) that had been intercepted by drift nets at two of the sites, and sequenced. The taxon proved to be Orthocladius pictipennis (Freeman, 1959), and not an undescribed taxon as was originally expected.

The northern hemisphere genus name Orthocladius v.d. Wulp, the base for the subfamily Orthocladiinae, had been used widely for a diverse assemblage of taxa, including those from the southern hemisphere. With a growing understanding of the immature stages, Orthocladius now is restricted to northern latitudes where it remains a diverse grouping. For New Zealand, the name was applied to three taxa by Freeman (1959): ‘Dactylocladus’ commensalis Tonnoir, ‘Orthocladius’ publicus Hutton and Orthocladius pictipennis Freeman. The first named is placed now in a New Zealand monotypic and endemic genus Tommirocladius (Cranston, 2007) related to New Zealand endemic Naonella Boothroyd and only far-distantly related to Orthocladius. As stated by Freeman (1959: 418) ‘O. publicus’ cannot be recognised from Hutton’s description or from re-examination of material. The remaining taxon, O. pictipennis, which was characterised by unusual dark pigmented wings, can now be assessed for its placement in Orthocladius based on our ability to associate the adult with its immature stages, and from study of its DNA.

In this paper we describe especially the immature stages, propose its phylogenetic relationships based on molecular analyses by the second author, and provide a generic name in recognition of Freeman’s studies.

MATERIALS AND METHODS

Collection methods emphasised kicking rocks to dislodge larvae from the benthos, and interception of drift for pupae, their exuviae and some larvae. Nets with a 300 µm mesh were exposed immediately downstream of kick sites, or for longer duration to intersect flowing water surfaces for up to 24 h. Association between larva, pupa and adult by rearing from live larvae was unsuccessful: pharate pupae form the basis of the pupal and adult description. Microscope slide preparation involved clearing, if necessary, with 10% KOH, neutralisation and initiation of dehydration with glacial acetic acid, then mounting from propan-2-ol (isopropanol) into Euparal. Larvae were decapitated and mounted in Hoyers and bodies sacrificed for DNA extraction. Pharate adults were extracted whole and subsequently their carcasses were permanently slide mounted as vouchers against their DNA. Attempts were made to remove the genitalia of the pharate adults from exuviae – neither were successful, and indeed both specimens became somewhat compressed laterally after DNA extraction and were unable to be orientated dorso-ventrally. Although this allowed examination of both dorsal and ventral half surfaces of the pupal abdomen, views of the male
hypopygium were suboptimal.

Extraction of total genomic DNA from tissues used the Qiagen DNeasy® Tissue Kit (Qiagen, Hilden, Germany) using the manufacturer’s protocol modified such that whole body tissue was digested overnight at 37°C and a final elution volume of 100 µL. A 732 bp fragment of the mtDNA COI gene, a 657 bp fragment of the nuclear 28S rDNA gene and two regions (CAD1 – 753 bp; CAD3 – 735 bp) of the nuclear protein-coding CAD (rudimentary) gene were amplified using published primer sequences (Table 1). Reactions were carried out in a total volume of 25 µL, containing 4 µL of template DNA, 0.6 µL of each primer (10pmol/µL – manufactured by Geneworks, Adelaide, Australia), 2.5 µL of 10X polymerase buffer (Roche, Mannheim, Germany), 3.0 µL of 25 mM MgCl₂ (Fisher, Perth, Australia), 2.0 µL of 10 mM dNTP’s (Roche), 0.2 µL of 5U/µL Taq polymerase (Roche). PCR protocols for all gene regions involved initial denaturing at 95°C for 4 mins and final extension at 72°C for 3 mins. Amplification of the COI region involved 39 cycles of 95°C for 30 s, 49°C for 1 min and 72°C for 1.5 min. The 28S region was amplified using a touchdown PCR cycle protocol beginning with 95°C for 30 s, 58°C for 1 min and 72°C for 1 min, and reducing the annealing temperature by 2°C every two cycles until it reached 42°C, which was then repeated 18 times. The cycle protocol used for CAD1 was 4 cycles of 95°C for 30 s, 51°C for 30 s and 72°C for 80 s, then 36 cycles using an annealing temperature of 45°C. CAD3 was amplified using 4 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 2 mins, then 6 cycles of 94°C for 30 s, 47°C for 1 min, 72°C for 2 mins, then 36 cycles of 94°C for 30 s, 42°C for 20 s and 72°C for 2.5 mins. Amplification products were purified using an UltraClean™ PCR Clean-up Kit (MoBio, Carlsbad, USA) following manufacturer’s guidelines. Purified PCR products were amplified using a standard ABI Big Dye® Terminator v.3.1 sequencing protocol and products were cleaned using a standard isopropanol precipitation protocol prior to sequencing at the Griffith University DNA Sequencing Facility (Nathan, Australia). Sequence electropherograms were edited manually using BioEdit Version 7.0.5 (Hall, 1999). All sequences are deposited in GenBank. (hq872707-11, hq87233-4, hq87281, hq872960-5)

Institutional Abbreviations. (ANIC) Australian National Insect Collection, Canberra, Australia; (NZAC) New Zealand Arthropod Collection, Auckland; (QUT) Queensland University of Technology, Brisbane; (ZSM) Zoologisches Staatssammlung, Munich, Germany.

**TAXONOMY**

**Paulfreemania Cranston & Krosch, gen. n.**

**Type species.** *Orthocladius pictipennis* Freeman, 1959 by designation and monotypy

**Diagnosis**

In the larval stage the combination in Orthocladiinae of palmate SI seta and ventromental beard is observed otherwise only in *Psectrocladius* and *Haloctadius (Psammocladius)*: neither are in the southern hemisphere, neither have paired premandibular teeth nor a nipple on the median mental tooth. The pupa, with spinose conjunctives on the dorsal and ventral abdomen resembles several austral orthoclad taxa, but the combination of lack of any thoracic horn and the consistent presence of an anal lobe with only 2 strong

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**Table 1. Primer sequences and references for the four gene regions analysed.**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'-3')</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: COI-s2183 (aka Jerry)</td>
<td>CAA CAT TTA TTT TGA TTT TTT GG</td>
<td>(Simon et al. 1994)</td>
</tr>
<tr>
<td>R: COI-a3014 (aka Pat)</td>
<td>TCC AAT GCA CTA ATC TGC CAT ATT A</td>
<td>(Simon et al. 1994)</td>
</tr>
<tr>
<td>F: 28S-s3660</td>
<td>GAG AGT TMA ASA GTA CGT GAA AC</td>
<td>(Dowton &amp; Austin 1998)</td>
</tr>
<tr>
<td>R: 28S-a335</td>
<td>TCG GAR GGA ACC AGC TAC TA</td>
<td>(Whiting et al. 1997)</td>
</tr>
<tr>
<td>F: CAD1 – 54F</td>
<td>GTN GTN TTY CAR ACN GGN ATG GT</td>
<td>(Moulton &amp; Wiegmann 2004)</td>
</tr>
<tr>
<td>R: CAD1 – 405R</td>
<td>GCN GTR TGY TCN GGR TGR AAY TG</td>
<td>(Moulton &amp; Wiegmann 2004)</td>
</tr>
<tr>
<td>F: CAD3 – 787F</td>
<td>GGD GTN ACN ACN GCN TGY TTY GAR CC</td>
<td>(Moulton &amp; Wiegmann 2004)</td>
</tr>
<tr>
<td>R: CAD3 – 1098R</td>
<td>TTN GGN AGY TGN CCN CCC AT</td>
<td>(Moulton &amp; Wiegmann 2004)</td>
</tr>
</tbody>
</table>
anal macrosetae is found otherwise only in one New Zealand species of *Stictocladius* Edwards (Cranston & Saether 2010). Adult orthoclads are notoriously difficult to identify other than by permutations of usually rather obscure features, but Freeman’s (1959) statement that in New Zealand the dark banded wing is unusual in orthoclads (other than this taxon and *Stictocladius*) remains correct. The simple gonostylus of *Paulfreemania* is distinguished from the bifid gonostyles of *Stictocladius*.

**Description**

**Adult.** Small, wing length c. 1.5 mm. Head with eye bare, almost rounded with slight dorsomedial extension. Temporal setation restricted to 1 outer postorbital. Clypeus broad, sparsely setose. Palp 5 segmented, consecutively longer from 2 to 5; 3rd segment without sensilla chaetica.

**Antenna.** With 13 flagellomeres, well-developed plume extending to apex which lacks a strong apical/subapical seta; groove extending from flagellomere 3 or 4 to 13; sensilla chaetica on flagellomeres 2-5 and sub-apex of 13; Antennal Ratio 0.38-0.40.

**Thorax.** Uniform medium brown. Antepronotum modestly developed, lobes strongly narrowed, medially separated. Thoracic setation: 2 lateral antepronotals; 2 small straight acrostichals in pale oval area of mid-scutum; 2-3 uniserial dorsocentrals each arising from pale oval area; 1-3 prealars; 4 scutellars.

**Wing.** Membrane dark pigmented in two broad bands (as Figured by Freeman 1959 plate 11i but less visible in pharate and unexpanded wing), with fine punctuation, without macrotrichia. Other features undetectable on pharate wing, except R with 2 setae, R4+5 with 1 strong seta. Squama setose (precise count unattainable).

**Legs.** With slender fore-tibial spur, longer than width of tibial apex, mid-tibia with two short spurs, one 75% length of the other; hind tibia with 1 short, 1 long spur, with sparse comb; mid- and hind spurs denticulate basally; pseudospurs absent. Sensilla chaetica apparently absent. Tarsomere 4 non-caudate, Pulvilli very short, empodium strong. Claws apically bifid.

**Tergites.** With few long setae arising from pale areas. Tergite IX microtrichiose, without setae.

**Hypopygium.** (Fig. 1). Tergite IX with no setae, anal point short (c 15 µm), basally microtrichiose, apically hyaline. Transverse sternapodeme and phallapodeme uninterpretable. Virga inverted U-shaped. Superior volsella completely absent; inferior volsella a more or less rectangular lobe. Gonostylus simple, apically tapering distally, with simple megaseta and without crista dorsalis.


**Antepronotum.** With 2 simple, very short, median antepronotals, without lateral antepronotal. Thoracic horn absent. Precorneals non-taeniate, very short, linear. Dorsocentrals short, fine, evenly-spaced, approximated, linear; 1 prealar present. Thorax smooth. Wing sheath bare, without pearls

**Abdomen.** (Fig. 2a, b). All tergites bare anterior to transverse spine rows; each successive tergite from II posteriorly with transverse spinule/tubercle row increasing in depth and size of spines on successive segments posteriorly. Tergite II without hookrow. Conjunctives of TIII - V with transverse multiserial rows of translucent, anteriorly-directed, spines/spinules, some of which may be hook-like. Sternite I bare, II and III with few median translucent spines, IV-VII with posterior transverse bands of spines, mostly uniserial, but with some smaller spinules involved especially more posteriorly; sternite VIII (of male) with fewer, shorter, translucent spines. Conjunctives of SIII – V with

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**Figure 1.** *Paulfreemania pictipennis* (Freeman). Male genitalia. A. Dorsal, left side.

**Figure 2.** *Paulfreemania pictipennis* (Freeman). Pupa. A, Lateral abdomen; B. Anal lobe.
multiserial transverse rows of fine spines/spinules (which are not hook-like). Pedes spurii A and B absent.

**Abdominal setation.** Not detectable due to pharate adult within. L setae short, non- taeniate.

**Anal lobe.** (Fig. 2b) rectangular, with sparse fine spinulation, with 2 subequal stout macrosetae, subequal in length to anal lobe, arising from rugose apex of anal lobe. Male genital sac rounded, extending posteriorly as far as end of anal lobe.

**Larva.** Head capsule (Fig. 3.1) length c. 300 μm. Dorsal head with S3 lying at slight narrowing of apotome, merging into an undivided anterior sclerite bearing S1 and S2, without suture; interpreted as forming a rugulose frontoclypeolabral apotome.

**Antenna.** (Fig. 3b) with 5 segments; segment 3 surrounded by Lauterborn organs and style reaching base of very short 4th segment, segment 5 as long as 3rd; no trace of any 6th. Antennal ratio about 1.2-1.6. Ring organ large, in basal quarter, setal scar at c 60% from base. Blade equal to length of 2nd segment, extending to base of 3rd segment, with short subsidiary blade.

**Labrum.** (Fig. 3c) with palmate SI with 6-9 tapering lobes of unequal sizes, SII lanceolate, SIII simple, short, weak, SIV seemingly absent. Labral lamella absent. Innermost chaeta pectinate, remainder simple, short. Pecten epipharyngis consisting of 3 simple lobes. Chaetulae laterales include 2 pectinate chaetulae; chaetulae basales apparently all simple. Premandible with double apical teeth, without brush.

**Mentum.** (Fig. 3d, e) with single median tooth with central ‘nipple’ (Fig. 3d) (often worn down to rounded tooth, Figure 3e), with 5 pairs of lateral teeth decreasing on an even slope. Ventromental plate strong, near semi-lunar, covering all lateral teeth in flattened mentum, with no connection to mid-mentum; with strong ventromental beard of flexible slightly taeniate, simple setae extending well beyond margin of plate. Setae submenti (SSm) anterior to base of mentum. Genal cephalic setae S9 and S10 close together, dorsal pit (DP) large and hyaline.

**Mandible.** (Fig. 3f) with apical tooth shorter than combined width of 4 inner teeth (innermost delimited, but not strongly separated from mola). Seta subdentalis lanceolate, without apical hook, extending to 3rd inner tooth. Seta interna a brush of 5 apically serrate branches.

**Body.** Unknown beyond anterior parapods which has serrate claws. Specific larval measurements (n=8 and units = μm, except where stated). Head length 3.0 mm (n = 3); postmentum length 110-125. Antenna 30-35, 11-14, 4-5, 1.5-2.0; 2-4, AR 1.2-1.6. Ring organ at 5-10, setal mark at 17-23. Blade 12-15. Mentum width 88-100. Mandible length 75-102.

**Etymology.** The derivation of the generic name commemorates Paul Freeman (1916-2010), whose studies on New Zealand and Australian Chironomidae laid the foundation for a modern understanding of the Australasian biota. The first author acknowledges with gratitude the mentorship of this important entomologist who was Keeper (head) of the Entomology Department at the British Museum (Natural History) during his early career.

**Material examined**

All specimens are deposited in QUT/ANIC unless otherwise stated. NEW ZEALAND: Buller (BR): L, 41°48.24’S 172°50.45’E, nr Lake Rotoiti, Borlase Ck, 678m, 13.vi.2007, P (molecular voucher NZ08 7.8), 22.xii.2008 (NZAC); 2L, P (molecular voucher NZ07 6.1), 41°46.50’S 172°46.30’E, Speargrass Ck, 562m, 13.vi.2007 (ZSM); L, 41°41.41’S 172°37.02’E, Hope River, 567m, 14.vi.2007; L, 41°41.13’S 172°27.09’E, Owen River, 291m, 15.vi.2007; 33L, 41°48.11’S 172°32.31’E, unnamed creek nr Lake Rotoroa (referred to here as Rotoroa Ck.), 675m, 15.vi.2007; 2L, 42°22.17’S 172°16.01’E, Jackson Ck, 500m, 1.vi.2007.

**Ecological notes**

The distribution and abundance of *Paulfreemania pictipennis* seems rather unusual. Although encountered in 6 stream locations in the catchment of the Buller River (Tasman-Nelson region), only in an unnamed creek (term Rotoroa Ck.) were...
more than 1-2 specimens found on any sampling occasion. Very unusually, pupal exuviae were completely absent despite geographically broad and locally intense sampling, using exposed drift nets at many sites during the past decade including those in the Buller drainage where larvae have been found. Evidence for the pupal-adult association came fortuitously from only two pharate males, from amongst many hundreds of samples. This counters nearly all previous experience –open drift nets ‘sample’ very efficiently the floating pupal exuviae left after upstream emergence of adults at the water surface. Inevitably this sampling of aquatic biodiversity is highly representative compared to larval sampling: behaviourally cryptic taxa can be missed by standard kick collections. Although tightly synchronised emergence may explain apparent absence for much of the year, collection in June (midwinter) and December (midsummer) of pharates undermines this explanation. More likely, the larvae may simply have been unrecognised previously.

### Phylogenetic placement and molecular association

Representatives of *Paulfreemania pictipennis* sequenced by the second author as part of a broader molecular phylogenetic assessment of the evolution of Gondwanan orthoclads (Krosch *et al.* in press, as *Orthocladius* *pictipennis*) placed the taxon as sister to the formally undescribed Australian taxon ‘genus Australia’ (Cranston 1996). Statistical support for this placement was high; the Bayesian (B) posterior probability was 1.00 and maximum likelihood (ML) bootstrap support was 100. These two taxa were nested within a broader, well-supported clade comprising *Naonella, Tonomoiracladius* and an undescribed taxon from Chile (B: 1.00; ML: 96). Associations were made between larval and pupal life stages based on identical COI sequences (data not shown). Numerous nucleotide substitutions were recorded between a single larva from Speargras Creek and all other representatives. This specimen was up to 5.8% divergent across all loci from the other sequenced members of the species, suggesting representatives. This specimen was up to 5.8% divergent across all loci from the other sequenced members of the species, suggesting more evolutionary distance than what might be expected.

### ACKNOWLEDGEMENTS

We are grateful to Bev Freer, Nelson-Marlborough Office of Department of Conservation (Te Papa Atawhai), for efficient and friendly handling, and renewal, of permit NM-20967-FAU to collect these aquatic insects, and thank Dr. Andrew Baker especially for assistance with midwinter sample collection and sorting. Travel expenses came from the Evert Schlinger endowment to the University of California, Davis to support the personal chair of the first author, in systematic entomology. The second author acknowledges the support for his PhD program (of which this forms a part) by Queensland University of Technology.

### REFERENCES


**Cranston PS. 2007.** The identity of *Dactylocladius commensalis* (Diptera: Chironomidae) revealed. *Aquatic Insects* 29: 104–113.


