Molecular phylogeny and morphology of *Pseudomallada* (Neuroptera: Chrysopidae), one of the largest genera within Chrysopidae

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Pseudomallada, comprising at least 166 described species, is not only one of the most species-rich genera within the green lacewing family Chrysopidae but also one of the largest within the entire order Neuroptera. Using external morphology, it has not been possible to parse *Pseudomallada* into smaller taxonomic units such as subgenera or species-groups. Here, we examine morphology, life-history traits, and nuclear DNA sequences in 49 described or putative species of *Pseudomallada* from all over the world, including 2 closely related species currently assigned to *Apertochrysa*. On the one hand, we find that body and egg coloration, as well as the mode of egg deposition, have evolved independently within different genetic clusters of species in response to environmental conditions favouring cryptic behaviour or sibling cannibalism. However, three lineages supported by molecular phylogenetic analysis are congruent with three clusters based on the morphology of the gonapophyses in the male genitalia; these comprise 29 of the taxa under study (59%). Seventeen additional species cluster in a *flavifrons*-group based on gonapophyses shape, but this cluster is not supported by molecular data. Three molecular singletons were identified, two of which can be assigned to one of the four gonapophyses groups. It is clear that at least *Apertochrysa eurydera*, and probably *A. edwardsi*, should be moved into the genus *Pseudomallada*, based on molecular data and the shape of the gonapophyses.

ADDITIONAL KEYWORDS: body colour – egg colour – morphology – oviposition – phylogeny – systematics – taxonomy.

INTRODUCTION

A detailed account of the actively changing history of the genus *Pseudomallada* Tsukaguchi was given by Dong *et al.* (2004). Navás (1925) established the genus *Mallada* and Nakahara (1955) the genus *Anisochrysa*, but at those times, the known species of the present genus *Pseudomallada* were still mostly called *Chrysopa* Leach. Adams (1975) realized that *Anisochrysa* was a synonym of *Mallada*, but for many years, the genus *Anisochrysa* was the home for

various genera, including all present species of the genus *Pseudomallada*, as well as *Mallada* and sometimes even *Chrysoperla* Steinmann. Brooks & Barnard (1990) used the genus name *Mallada* for the present genera *Mallada* and *Pseudomallada*, whereas Yang (1991) separated *Mallada* and coined the new genus name *Dichochrysa*. Brooks (1994) considered the new genus *Dichochrysa* to be the sister taxon to all other Chrysopini. In 1995, Tsukaguchi (1995) changed the genus name *Dichochrysa* to *Pseudomallada*. A subsequent petition to the International Commission on Zoological Nomenclature to conserve the genus name *Dichochrysa* was not approved, thus validating

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the name *Pseudomallada* for species formerly placed in *Dichochrysa* (Anonymous, 2010). The Lacewing Digital Library (LDL) (<http://lacewing.tamu.edu/>) currently includes at least 166 described species of *Pseudomallada*, making this not only one of the largest genera in the Chrysopidae but also one of the largest in the entire order Neuroptera.

With the help of molecular sequence data, the phylogenetic positions of some *Pseudomallada* species (still as *Dichochrysa*) within the family Chrysopidae were inferred by Winterton & de Freitas (2006) and Haruyama *et al.* (2008). Its closest relatives among the Chrysopini were hypothesized to be *Glenochrysa* Esben-Petersen, *Chrysopa*, and *Plesiochrysa* Adams. Consequently, we have good evidence that *Mallada* s.s. and *Pseudomallada* are distant rather than close relatives, that *Mallada* is closely related to *Chrysoperla*, and that *Pseudomallada* as previously construed is not the sister taxon of all other Chrysopini.

In the present study, we tested the hypothesis that *Pseudomallada* is a monophyletic taxon (see Aspöck, Hölzel & Aspöck, 2001) by examining nuclear DNA sequence data, genital morphology, body coloration, egg colour, and oviposition behaviour in 49 described or putative species of the genus from around the world. A principal goal was to find unique characters and patterns of sequence data shared by the numerous and often similar-looking species thought to comprise this giant taxon.

MATERIAL AND METHODS

MOLECULAR METHODS

For the molecular phylogenetic analysis, 85 neuropteran taxa were used, including a subset of the chrysopid species reported in Duelli, Henry & Mochizuki (2014). *Micromus linearis* Hagen (Neuroptera: Hemerobiidae) served as the out-group (Table 1). Because we suspect a complex of cryptic species exists within *Pseudomallada* *prasinus* and *P. abdominalis*, we chose to analyze three individuals of *P. prasinus* and two individuals of *P. abdominalis* from different localities. Describing authors of each named species are given in Table 1.

Total genomic DNA was extracted from the thorax using a QuickGene DNA tissue kit S (WAKO, Tokyo, Japan) following the manufacturer's instructions. The thorax was removed from the specimen using a utility knife, homogenized with a plastic micro-pestle in extracting buffer (MDT + EDT), and incubated for 24–48 h at 55 °C.

Portions of the nuclear genes phosphoenolpyruvate carboxykinase (*PepCK*), wingless (*wg*), and sodium/potassium ATPase alpha subunit (*ATPase*) were amplified by the same primers and PCR protocols as specified in a previous paper (Haruyama *et al.*, 2008). The PCR reaction typically contained 1xLA Taq buffer

(Mg⁺⁺ free), 3 mM MgCl₂, 0.4 mM dNTP mixture, 1 mM of each primer, 1.25 units of TaKaRa Ex Taq, and 1 µL of DNA solution.

Nested PCR protocols were performed for specimens that were difficult to amplify with these primers. Wide-range primer sets were used for the first PCR: PEPCK-mmF/PEPCK-wfR for the *PepCK* gene, WG1F2/LepWG2a for the *wg* gene, and SodF13/ SodR13 for 410 bp of the *ATPase* gene were used (Table 2). The amplification program was: 94 °C (1 min); 30 cycles of 94 °C (30 s), 50 °C (30 s), and 72 °C (2 min). The PCR reaction typically contained 1xLA Taq buffer (Mg⁺⁺ free), 3 mM MgCl₂, 0.4 mM dNTP mixture, 1 mM of each primer, 1.25 units of TaKaRa Ex Taq, and 1 µL of DNA solution.

Following that, we diluted the first PCR product 50-fold with Double Distilled Water (DDW) and used 1 µL of the dilution solution as a template for the second PCR. The second PCR was performed using internal primer sets as follows: 511.2drc/19.5dF for the *PepCK* gene, WG1-wm/WG2-2R and LepWG1/WG2.1 for the *wg* gene, and SodinF/SodinR for 410 bp of the *ATPase* gene (Table 2). The *wg* gene was amplified by dividing it into two fragments.

The amplification program was as follows: 94 °C (4 min); 4 cycles of 94 °C (30 s), 51 °C (30 s), and 72 °C (1 min 20 s); 36 cycles of 94 °C (30 s), 45 °C (30 s), and 72 °C (1 min 20 s); 72 °C (3 min). The PCR reaction mixture contained 1xEx Taq buffer, 0.4 mM dNTP mixture, 1 mM of each primer, 1.25 units of TaKaRa Ex Taq, and 1 µL of DNA solution.

We succeeded in amplifying the upstream region of the 410 bp *ATPase* gene fragment. To do this, a Sod70F/ SodC170R primer set was used for the first PCR and a Sod150F/Sod90Rm was used for the second PCR. Amplification programs and contents of the reaction mixture were the same as for the other genes.

PCR products were checked using 1% agarose gel electrophoresis and purified by NucleoSpin Gel and PCR Clean-up (Takara Bio Inc., Otsu, Japan). If nonspecific bands were found, specific bands were excised prior to purification. The purified products were submitted to Greiner Bio-One Co. Ltd (Tokyo, Japan) for direct sequencing. Sequences were assembled using GENETYX-MAC v15.0 (Genetyx Corporation, Tokyo, Japan). The *wg* gene sequence was obtained by assembling the sequences of two PCR fragments (WG1-wm/WG2-2R and LepWG1/WG2.1). Nine hundred and eighteen base pairs of *ATPase* gene sequence were obtained by assembling the sequences of the 410 bp *ATPase* gene and of the Sod150F/Sod90Rm PCR fragment. Accession numbers for the three nuclear gene sequences are given in Table 1.

MOLECULAR PHYLOGENETIC ANALYSIS

Phylogenetic trees were estimated using maximum likelihood (ML) and Bayesian inference (BI) on

Table 1. List of specimens of Hemerobiidae and Chrysopidae used in this study, including accession numbers

Taxon	DDBJ accession number			Collection data
	<i>PepCK</i>	<i>wg</i>	<i>ATPase</i>	
Hemerobiidae				
<i>Micromus linearis</i> Hagen	AB287526	AB287527	AB287528	Japan, Chiba, 23 V. 2004, N. Haruyama
Chrysopidae				
Nothochrysinae				
<i>Kimochrysa africana</i> (Kimmins)	AB287888	AB287889	AB287890	RSA, Bushmans Kloof, 5 X. 2004, P. Duelli
Apochrysinae				
<i>Anapochrysa voeltzkowi</i> (Weele)	AB287882	AB287883	AB287884	RSA, Tsitsikamma, 18 II. 2001, P. Duelli
<i>Apochrysa matsumurae</i> (Okamoto)	AB287532	AB287533	AB287534	Japan, Chiba, 19 X. 2003, N. Haruyama
Chrysopinae				
Ankylopterygini				
<i>Ankylopteryx gracilis</i> Nakahara	AB287915	AB287916	AB287917	Japan, Okinawa, 14 XI. 2006, I. Aoki
<i>Ankylopteryx octopunctata</i> (Fabricius)	AB287538	AB287539	AB287540	Japan, Okinawa, 19 III. 2004, N. Haruyama
<i>Semachrysa matsumurae</i> (Okamoto)	AB287547	AB287548	AB287549	Japan, Okinawa, 19 III. 2004, N. Haruyama
Belonopterygini				
<i>Italochrysa italica</i> (Rossi)	AB287553	AB287554	AB287555	France, Le Muy, 9 VIII. 1996, P. Duelli
<i>Italochrysa nigrovenosa</i> Kuwayama	AB287559	AB287560	AB287561	Japan, Osaka, 9 VII. 2005, N. Haruyama
Chrysopini				
<i>Apertochrysa edwardsi</i> (Banks)	LC102755	LC102780	LC102805	Australia, Myara, WA, 15 XI. 2000, J. B. Johnson and P. Duelli
<i>Apertochrysa eurydera</i> (Navás)	LC110411	LC110412	LC110413	Ghana, Lake Bosumtwé, 13 IV. 2007, P. Duelli
<i>Atlantochrysa atlantica</i> (McLachlan)	AB820321	AB820322	AB820323	Madeira, Porto Moniz, Levada Janela, 11 IX. 2001, P. Duelli
<i>Brinckochrysa kintoki</i> (Okamoto)	AB287574	AB287575	AB287576	Japan, Chiba, 20 IX. 2004, N. Haruyama
<i>Brinckochrysa turkanensis</i> (Navás)	AB287580	AB287581	AB287582	RSA, WCP, Cederberg, 2 X. 2004, P. Duelli
<i>Chrysemosa jeanneli</i> (Navás)	AB287583	AB287584	AB287585	RSA, WCP, Cederberg, 22 II. 2001, P. Duelli
<i>Chrysopa dorsalis</i> Burmeister	AB287589	AB287590	AB287591	Spain, Ondarroa, 17 VI. 2006, P. Duelli
<i>Chrysopa viridana</i> Schneider	AB287631	AB287632	AB287633	Italy, Ferrara, 21 VI. 2005, P. Duelli
<i>Chrysoperla carnea</i> (Stephens)	AB287643	AB287644	AB287645	Germany, commercial strain
<i>Chrysoperla comans</i> (Tjeder)	AB287903	AB287904	AB287905	RSA, Drakensberg, Clarens, 15 II. 2002, P. Duelli
<i>Chrysoperla furcifera</i> (Okamoto)	AB287652	AB287653	AB287654	Japan, Chiba, 9 IX. 2003, N. Haruyama
<i>Chrysoperla pudica</i> (Navás)	AB287673	AB287674	AB287675	RSA, Cederberg, 2 X. 2004, P. Duelli

Table 1. Continued

Taxon	DDBJ accession number			Collection data
	<i>PepCK</i>	<i>wg</i>	<i>ATPase</i>	
<i>Chrysotropia ciliata</i> (Wesmael)	AB287685	AB287686	AB287687	Japan, Yamanashi, 7 VIII. 2004, A. Mochizuki
<i>Cunctochrysa albolineata</i> (Killington)	AB287700	AB287701	AB287702	France, Lourdes, 19 VI. 2006, P. Duelli
<i>Cunctochrysa kannemeyeri</i> (Esben-Petersen)	AB287891	AB287892	AB287893	RSA, WCP, Wolfdrift, 1 X. 2004, P. Duelli
<i>Cuctochrysa baetica</i> (Hölzel)	AB820324	AB820325	AB820326	France, Carcès VAR, 23 VII. 2004, P. Duelli
<i>Mallada basalis</i> (Walker)	AB287808	AB287809	AB287810	Taiwan, lab-cultured strain
<i>Mallada desjardinsi</i> Navás	AB287811	AB287812	AB287813	Japan, Chiba, 10 VIII. 2003, N. Haruyama
<i>Mallada krakatauensis</i> (Tsukaguchi)	AB287826	AB287827	AB287828	Japan, Hokkaido, 19 VIII. 2005, N. Haruyama
<i>Meleoma arizonensis</i> (Banks)	AB287832	AB294231	AB287833	USA, Arizona, 7 VIII. 2003, P. Duelli
<i>Meleoma furcata</i> (Banks)	AB287834	AB287835	AB287836	USA, Arizona, 5 VIII. 2003, P. Duelli
<i>Meleoma titschacki</i> Navás	LC104976	LC104977	LC104978	Nicaragua, Selva Negra, 22 X. 2009, P. Duelli, U. Aspöck, and H. Aspöck
<i>Nineta itoi</i> Tsukaguchi	AB287846	AB287847	AB287848	Japan, Yamanashi, 7 VIII. 2004, A. Mochizuki
<i>Nineta pallida</i> (Schneider)	AB287852	AB287853	AB287854	Switzerland, Zürich, 19 VII. 2006, P. Duelli
<i>Peyrimhoffina gracilis</i> (Schneider)	AB287867	AB287868	AB287869	Switzerland, Zürich, 27 VII. 2006, P. Duelli
<i>Plesiochrysa lacciperda</i> (Kimmins)	AB287870	AB287871	AB287872	India, cultured strain
<i>Plesiochrysa ramburi</i> (Schneider)	AB294225	AB294226	AB294227	Australia, Chichester, 8 XI. 2000, P. Duelli
<i>Pseudomallada abdominalis</i> (Brauer) (Switzerland)	LC102731	LC102756	LC102781	Switzerland, Agarone, 17 V. 2015, P. Duelli
<i>Pseudomallada abdominalis</i> (Brauer) (Italy)	LC102732	LC102757	LC102782	Italy, Cisternino, 30 VI. 2015, P. Duelli
<i>Pseudomallada alcestes</i> (Banks)	AB287709	AB287710	AB287711	Japan, Okinawa, 17 III. 2004, N. Haruyama
<i>Pseudomallada astur</i> (Banks)	AB287715	AB287716	AB287717	Japan, Okinawa, VII. 2005, I. Aoki
<i>Pseudomallada baronissus</i> (Navás)	AB287718	AB287719	AB287720	RSA, Drakensberg, Clarens, 13 II. 2002, P. Duelli
<i>Pseudomallada benedictae</i> (Séméria)	LC102733	LC102758	LC102783	France, Palaysan, 9 VIII. 2006, P. Duelli
<i>Pseudomallada chloris</i> (Schneider)	AB287721	AB287722	AB287723	RSA, Drakensberg, Clarens, 15 II. 2002, P. Duelli
<i>Pseudomallada clathratus</i> (Schneider)	LC102734	LC102759	LC102784	Switzerland, Agarone, 3 IX. 2013, P. Duelli
<i>Pseudomallada cognatellus</i> (Okamoto)	AB287733	AB287734	AB287735	Japan, Kagoshima, 17 X. 2005, N. Haruyama
<i>Pseudomallada duplicatus</i> (Navás)	LC102735	LC102760	LC102785	Mauritius, Nouvelle Decouverte, 11 XII. 2004, P. Duelli

Table 1. Continued

Taxon	DDBJ accession number			Collection data
	<i>PepCK</i>	<i>wg</i>	<i>ATPase</i>	
<i>Pseudomallada flavifrons</i> (Brauer)	AB287736	AB287737	AB287738	Italy, Ferrara, 21 VI. 2005, P. Duelli
<i>Pseudomallada formosanus</i> (Matsumura)	AB287739	AB287740	AB287741	Japan, Chiba, 11 IX. 2003, N. Haruyama
<i>Pseudomallada genei</i> (Rambur)	AB287894	AB287895	AB287896	Spain, Andalusia, 2 IX. 2001, P. Duelli
<i>Pseudomallada gunvorae</i> (Tjeder)	LC102736	LC102761	LC102786	RSA, Pretoria, 17 II. 2002, P. Duelli
<i>Pseudomallada hamatus</i> (Tjeder)	LC102737	LC102762	LC102787	RSA, Drakensberg, Mont-Aux-Sources, 28 III. 2015, P. Duelli, J. B. Johnson, and M. W. Mansell
<i>Pseudomallada handschini</i> (Navás)	LC102738	LC102763	LC102788	RSA, Tendele Camp, 13 II. 2002, P. Duelli
<i>Pseudomallada ibericus</i> (Navás)	AB287745	AB287746	AB287747	Spain, Andalusia Jaizkibel, 14 VI. 2006, P. Duelli
<i>Pseudomallada inopinatus</i> (Hölzel and Ohm)	LC102739	LC102764	LC102789	France, La Réunion, 28 IX. 2004, P. Duelli
<i>Pseudomallada inornatus</i> (Navás)	LC102740	LC102765	LC102790	France, Prades, 13 VII. 2015, P. Duelli
<i>Pseudomallada karoensis</i> (Hölzel)	AB287748	AB287749	AB287750	RSA, WCP, Wolfdrift, 2 X. 2004, P. Duelli
<i>Pseudomallada kibonotoensis</i> (van der Weele)	LC102741	LC102766	LC102791	RSA, Soutpansberg, Limpopo, 22 III. 2015, P. Duelli, J. B. Johnson, and M. W. Mansell
<i>Pseudomallada luaboensis</i> (Tjeder)	LC102742	LC102767	LC102792	Ghana, Cape Coast, 8 IV. 2007, U. Aspöck and H. Aspöck
<i>Pseudomallada luctuosus</i> (Banks)	AB287751	AB287752	AB287753	USA, Texas, 2 VIII. 2003, P. Duelli
<i>Pseudomallada macleodi</i> (Adams and Garland)	LC102743	LC102768	LC102793	USA, Arizona, 7 VIII. 2003, P. Duelli
<i>Pseudomallada marianus</i> (Navás)	LC102744	LC102769	LC102794	Switzerland, Agarone, 15 IX. 2015, P. Duelli
<i>Pseudomallada</i> sp. gray	LC102746	LC102771	LC102796	RSA, WCP, Cederberg, 2 X. 2005, P. Duelli
<i>Pseudomallada</i> sp. Sodere	LC102747	LC102772	LC102797	Ethiopia, Sodere, 26 IV. 2003, P. Duelli
<i>Pseudomallada nicolainus</i> (Navás)	AB287757	AB287758	AB287759	RSA, Kuruman, 29 IX. 2004, P. Duelli
<i>Pseudomallada myassalandicus</i> (Navás)	LC102748	LC102773	LC102798	RSA, Soutpansberg, Limpopo, 22 III. 2015, P. Duelli, J. B. Johnson, and M. W. Mansell
<i>Pseudomallada parabolus</i> (Okamoto)	AB287760	AB287761	AB287762	Japan, Iwate, 7 VIII. 2004, N. Haruyama
<i>Pseudomallada perfectus</i> (Banks)	AB287766	AB287767	AB287768	USA, Arizona, 5 VIII. 2003, P. Duelli
<i>Pseudomallada perpallidus</i> (Tjeder)	AB287769	AB287770	AB287771	RSA, Drakensberg, Clarens, 16 II. 2002, P. Duelli

Table 1. Continued

Taxon	DDBJ accession number			Collection data
	<i>PepCK</i>	<i>wg</i>	<i>ATPase</i>	
<i>Pseudomallada picteti</i> (McLachlan)	LC102749	LC102774	LC102799	Spain, Puenteclasa, 19 VI. 2007, P. Duelli
<i>Pseudomallada prasinus</i> (Burmeister) (Japan)	AB287775	AB287776	AB287777	Japan, Nagano, 9 VIII. 2003, N. Haruyama
<i>Pseudomallada prasinus</i> (Burmeister) (Greece)	LC102750	LC102775	LC102800	Greece, lab rearing, M. L. Pappas
<i>Pseudomallada prasinus</i> (Burmeister) (Switzerland)	AB287778	AB287779	AB287780	Switzerland, Leuk, 3 V. 2006, P. Duelli
<i>Pseudomallada pulchrinus</i> (Tjeder)	LC102751	LC102776	LC102801	RSA, Soutpansberg, 21 III. 2015, P. Duelli
<i>Pseudomallada rubicundus</i> (Hölzel)	AB287781	AB287782	AB287783	RSA, WCP, Gamkaskloof, 15 II. 2001, P. Duelli
<i>Pseudomallada sjostedti</i> (van der Weele)	AB287897	AB287898	AB287899	Ethiopia, Wendo Genet, 13 IV. 2003, P. Duelli
<i>Pseudomallada</i> sp.1	AB287784	AB287785	AB287786	Japan, Okinawa, VI. 2005, I. Aoki
<i>Pseudomallada</i> sp.2	AB287787	AB287788	AB287789	Japan, Okinawa, VI. 2005, I. Aoki
<i>Pseudomallada spissinervis</i> (Tjeder)	LC102752	LC102777	LC102802	RSA, WCP, Gamkaskloof, 14 II. 2001, P. Duelli
<i>Pseudomallada tactus</i> (Navás)	AB287790	AB287791	AB287792	RSA, WCP, Wolfdrift, 2 X. 2004, P. Duelli
<i>Pseudomallada ussuriensis</i> (Makarkin)	AB287802	AB287803	AB287804	Japan, Iwate, 7 VIII. 2004, N. Haruyama
<i>Pseudomallada venosus</i> (Rambur)	LC102753	LC102778	LC102803	Georgia, Vardzia, 15 VIII. 2013, D. Bolt and P. Duelli
<i>Pseudomallada ventralis</i> (Curtis)	LC102754	LC102779	LC102804	Switzerland, Agarone, 10 IX. 2008, P. Duelli
<i>Pseudomallada zelleri</i> (Schneider)	AB287805	AB287806	AB287807	Switzerland, Agarone, 14 V. 1999, P. Duelli
<i>Suarius walsinghami</i> Navás	AB287873	AB287874	AB287875	Spain, Alhaurin de la Torre, 2 IX. 2001, P. Duelli
<i>Yumachrysa apache</i> (Banks)	AB287876	AB287877	AB287878	USA, New Mexico, 13 VIII. 2003, P. Duelli

ATPase, sodium/potassium ATPase alpha subunit gene; *PepCK*, phosphoenolpyruvate carboxykinase gene; *wg*, wingless gene.

combined DNA sequence data. Alignment of protein-encoding genes was inferred manually with reference to translated amino acid sequences using MUSCLE (Edgar, 2004a, b) in MEGA v6.06 (Tamura *et al.*, 2013). Some amino acid insertions and deletions were found, but only in wingless (*wg*) gene sequences.

The alignment for the combined DNA data was partitioned by codon position for each gene and a separate partition-specific substitution model was determined using the greedy algorithm in PARTITIONFINDER v1.1 (Lanfear *et al.*, 2012). The best partitioning scheme and substitution models are given in Table 3.

ML analyses were performed using RAXML (Stamatakis, 2014). The ML tree was generated using

1000 bootstrap replicates to evaluate statistical confidence at each branch (Felsenstein, 1985).

MRBAYES v3.2.1 (Ronquist *et al.*, 2012) was used for dedicated BI phylogenetic analysis of the data. The Markov chain Monte Carlo analysis ran for 6.2 million generations. The Average Standard Deviation of Split Frequencies was 0.007 at the end of the analysis. Trees were sampled every 500 generations, discarding the first 25% of trees as burn-in. We confirmed with TRACER v1.6 (Rambaut *et al.*, 2014) that Estimated Sample Size for all parameters was more than 200. In the BI analysis, posterior probabilities (PP) were calculated to evaluate statistical confidence at each node.

Table 2. Primers used for nested PCR to extract and amplify DNA from specimens listed in Table 1

Gene	Primer name	Sequence (5'–3')	Reference
Wide ranged primer			
<i>PepCK</i>	PEPCK-mmF	TGYGGNAARACNAAYYTNGCNATGATG	Brower & DeSalle (1998) Haruyama <i>et al.</i> (2008) Haruyama <i>et al.</i> (2008)
	PEPCK-wfR	TCNGCRTCNGCRTCYYTTCNKRAACCA	
<i>wg</i>	WG1F2	CARGARTGYAARTGYCAYGGNATG	
	LepWG2a	ACTICGCARCACCARTGGAATGTRCA	
<i>ATPase</i>	SodF13	CTATGGTTTRGATGCCGTTATCTT	
	SodR13	GGAATTTTCRCANACTTTCTT	
	Sod70F	CCNCCNAARCARACNCCNGARTGGTA	
	SodC170R	TCRATRATTTGRTTTRTCRAACCACAT	
Internal primer			
<i>PepCK</i>	511.2drc	GGMCGCATTGCRAAYGGRTCGTGCAT	Modified a sequence of Friedlander <i>et al.</i> (1996)
	19.5dF	GGNGAYGAYATIGCBTGGATG	Friedlander <i>et al.</i> (1996)
<i>wg</i>	WG1-wm	TGYACNGTNAARACNTGYTGGATG	Brower & DeSalle (1998)
	WG2-2R	TTRCAYTTNACYTCRCARCACCA	
	LepWG1	GARTGYAARTGYCAYGGYATGTCTGG	
	WG2.1	CGATAHCCHCGNCCRCAACACA	
<i>ATPase</i>	SodinF	CTTCCTTATTGGTATCATTGTAGC	
	SodinR	CGCGACTTTCTTGTTGCGYTTACG	
	Sod150F	ATHATGGARWSNNGNAARAAYATGGT	
	Sod90Rm	GARCCYARNGTTTCYACAGCTTC	

ATPase, sodium/potassium ATPase alpha subunit gene; *PepCK*, phosphoenolpyruvate carboxykinase gene; *wg*, wingless gene. B = C or G or T, H = A or C or T, M = A or C, N = A or C or G or T, K = G or T, R = A or G, S = G or C, W = A or T, Y = T or C, I = inosine.

Evolutionary divergences among selected taxa and clades of taxa were estimated as the number of base substitutions per site averaged over all sequence pairs between the taxa or clades. Method of analysis was uncorrected *P*-distances as implemented in MEGA v6.06.

GENITAL MORPHOLOGY

Published drawings of male genitalia were available for most of the taxa sequenced. The most important sources were Tjeder (1966), Tsukaguchi (1995), Aspöck, Aspöck, & Hölzel (1980), Hölzel (1993), Hölzel & Ohm (1995, 2000), Adams & Garland (1982), New (1980), and Brooks & Barnard (1990). Many of the taxa were also available from the authors' collections and from fresh material collected for genetic analyses. Male abdomens of those species were kept in a warm KOH solution for several hours and then dissected and viewed under a Leica APO binocular microscope (×80–×800). The male genitalia generally used for species descriptions in the genus *Pseudomallada* are the gonarcus with the arcessus, gonapsis, and tignum.

BODY AND EGG COLORATION AND MODE OF OVIPOSITION

While the colour of the body was known in most species treated here, only for less than half of the

species had the mode of egg deposition been previously described in the literature. Most species were reared in lab cultures to obtain information on their eggs and larval stages. Moreover, the offspring could be used for sequence analysis and to obtain information on male genitalic morphology.

The following criteria were used to describe body coloration, egg colour, and mode of egg deposition:

Body coloration

Most *Pseudomallada* species are basically green, marked in Figure 1 by green rectangles. Some are bright green (Fig. 2A), others bluish green, and some have brown markings or bands on the thorax, but as long as the general aspect is green, they are listed here as green. A minority of species, mostly from hot and dry habitats, are grey or brown without any green (see Fig. 2B). They are listed as grey here and marked in Figure 1 by yellow rectangles.

Egg colour

Egg colour was recorded 1 day after oviposition, under full-spectrum illumination (daylight). Eggs can be plain green, light green, or bluish green (green circles in Fig. 1) or white, beige, or grey (white circles in Fig. 1).

Table 3. Optimal data partitions and best nucleotide substitution models selected by PARTITIONFINDER v1.1 (Lanfear *et al.*, 2012) to infer phylogenetic relationships of species listed in Table 1

Subset	Subset partitions (nuclear genes and codon positions)	Best model
1	<i>PepCK</i> pos.1	TIM + I + Γ
2	<i>PepCK</i> pos.2, <i>wg</i> pos.1, <i>wg</i> pos.2	HKY + I + Γ
3	<i>PepCK</i> pos.3, <i>ATPase</i> pos.3	HKY + I + Γ
4	<i>wg</i> pos.3	TVM + I + Γ
5	<i>ATPase</i> pos.1	GTR + I + Γ
6	<i>ATPase</i> pos.2	JC + I + Γ

ATPase, sodium/potassium ATPase alpha subunit gene; *PepCK*, phosphoenolpyruvate carboxykinase gene; *wg*, wingless gene.

Oviposition mode

Most *Pseudomallada* species deposit their eggs singly, on silken stalks. Other species in the genus exhibit bundled egg stalks (Fig. 2C). Some species deposit their eggs closely spaced in groups or in rows, but those are nevertheless treated here as laying single eggs. All known modes of oviposition in chrysopids are presented in Duelli (1984).

RESULTS

MOLECULAR PHYLOGENY

The combined DNA sequence length of the alignment was 1926 bp, comprising 483 bp of *PepCK*, 525 bp of *wg*, and 918 bp of *ATPase*.

Phylograms of Chrysopidae show the Bayesian tree (Supporting Information, Fig. S1) with nodes labelled by PP and the ML tree (Supporting Information, Fig. S2) with nodes labelled by bootstrap values. Only values of PP above 0.8 and of bootstraps above 50% are included.

Our DNA sequence data analysis united 47 *Pseudomallada* taxa and two *Apertochrysa* species, *A. eurydera* and *A. edwardsi*, in a single clade (PP = 0.99, ML bootstrap values = 89% for combined data). We call this clade *Pseudomallada s.l.* Sub-trees of the taxa within the *Pseudomallada s.l.* are shown in Figure 1.

The species in *Pseudomallada s.l.* were allocated to two clades. The first clade, P1, included *P. astur*, *P. alcestes*, two unknown Japanese species (*P. sp. 1* and *P. sp. 2*), *A. eurydera*, *P. cognatellus*, *P. parabolus*, *P. formosanus*, *P. ussuriensis*, *P. pulchrinus*, *P. duplicatus*, and *P. sjostedti* (PP = 1.00, bootstrap 80%). The second clade, P2, included all the remaining species except *A. edwardsi* and *P. inornatus* (PP = 1.00, bootstrap 84%). *Pseudomallada inornatus* was supported

as the sister taxon of the P2 clade (PP = 0.99, bootstrap 81%). The phylogenetic position of *A. edwardsi* within *Pseudomallada* was unresolved. The P2 clade was divided further into two sub-clades, P3 and P4. The P3 clade included *P. benedictae*, *P. zelleri*, *P. ventralis*, *P. prasinus*, *P. abdominalis*, and *P. marianus* (PP = 1.00, bootstrap 93%). The P4 clade included the rest of the species, except *A. edwardsi* and *P. inornatus* (PP = 1.00, bootstrap 96%). Moreover, *P. venosus*, *P. sp. Sodere*, *P. rubicundus*, *P. tactus*, *P. nicolainus*, *P. karoensis*, *P. genei*, and *P. clathratus* formed a well-supported additional clade, P5, within P4 (PP = 1.00, bootstrap 97%). *Pseudomallada inopinatus* appeared to be the sister taxon to the P5 clade (PP = 0.96, bootstrap 82%).

We then compared the molecular phylogenetic results with some morphological traits.

GENITAL MORPHOLOGY

Published figures and/or new drawings of male genital structures of 48 taxa were analyzed for quantifiable characters. For only one species (*P. sp. 1* from Japan), no genital structures were available, because only one unidentified female of that species was collected, and she laid no eggs.

The gonarcus, arcessus, and tignum are sclerotized structures just above the anus. The tignum was present in all nominal *Pseudomallada* species examined, but not in *A. eurydera* or *A. edwardsi*. It was a uniform structure throughout the genus and yielded no quantifiable measurements for sorting the species. The gonarcus and arcessus, on the other hand, are rather complex three-dimensional structures. However, they also were basically the same in most *Pseudomallada* species. Moreover, the quality of published drawings is too variable to make quantitative comparisons to previously described species. The situation is different for the gonapsis, a horizontal structure just below the anus, where clear differences between groups of species were discernible. All available graphics from literature and new preparations were sorted for visual similarity. Figure 3 shows the four icons to illustrate the general shape of the four main gonapsis types and the two measured structures we call here 'stem' and 'wings'.

G1: The synapomorphy of the 'dancer' shaped gonapsis (Fig. 3) is the frontally bifurcated 'stem'. The frontal 'wings' are broad, sometimes curled, and variable in size and shape. All taxa of the molecular phylogenetic clade P1 belonged to this 'dancer' group G1 (G for gonapsis). We call G1/P1 the *alcestes*-group, because *P. alcestes* is a well-known and widespread species exemplifying the group icon.

Figure 1. Maximum likelihood (ML) tree for *Pseudomallada* s.l. The tree is inferred from a combined analysis of three nuclear genes. The total sequence length of the alignment was 1926 bp, comprising 483 bp of *PepCK*, 525 bp of *wg*, and 918 bp of *ATPase*. Values placed on each node are ML bootstrap values $\geq 50\%$ /Bayesian posterior probabilities (PP) ≥ 0.80 . Dashes (–) mark branches with less than 0.80 PP or less than 50% bootstrap support. Body colour, egg colour and oviposition mode for each taxon are shown to the right of the tree. P1–P5: five major clades inferred from DNA sequences. G1–G4: four major gonapophyseal species-groups based on the general shape classes of male genitalia shown as icons in Figure 3.

Figure 2. Photographs from life of (A) green *Pseudomallada chloris* from South Africa, (B) grey/brown *Pseudomallada venosus* from Georgia, and (C) bundled egg pedicels of *P. venosus*.

Figure 3. Icons classifying the general shape of the gonapsis of the male genitalia of *Pseudomallada* s.l. into four distinct groups. Each of the four gonapsis species-groups is named after the most widespread or best known species in the group. Icon names are meant to simplify complex descriptions.

G2: The gonapsis of this group is similar to that of G3, but the 'wings' are broader in G2, reminiscent of the shape of a fruit bat (Fig. 3). At the base, they are at least twice as broad as the 'stem'. A synapomorphy of this group of species, congruent with the clade P3, is the black dot between the antennae. Some species from other groups may also exhibit an inter-antennal dot, but in those it was brown or reddish. We call G2/P3 the *prasinus*-group.

G3: The 'swift' icon (Fig. 3) in this group is characterized by slender 'wings'. At their base, they are less than twice as broad as the 'stem'. In some species, the 'wings' appeared to be covered by a veil, making them look broader. The group constitutes a part of the clade P4, but it is not supported by molecular data, nor did we find any clear synapomorphy uniting its members. We call G3 the *flavifrons*-group.

G4: The synapomorphy of the 'butterfly' shaped gonapsis (Fig. 3) is the unique appearance of the two wing pairs: the caudal 'wings' are always speckled and more rounded than the frontal ones. Group G4 is congruent with clade P5. We call G4/P5 the *venosus*-group.

The phylogenetic singleton *A. edwardsi* did not fit into any of the molecular phylogenetic clades. It is the only Australian species included in this study. It clearly belongs to *Pseudomallada* but lacks a tignum. However, the morphology of the gonapsis of *A. edwardsi* (Fig. 4A) was most similar to that seen in G1.

Another phylogenetic singleton is *P. inornatus*. The shape of the tiny gonapsis of *P. inornatus* (Fig. 4B) is markedly different from all four icons shown in Figure 3.

The gonapsis of *P. inopinatus* from the island of La Réunion (Fig. 4C) has only one pair of 'wings' and more of a 'swift' shape, resembling the condition seen in the *flavifrons*-group (G3).

BODY COLORATION

Of the 49 taxa examined for their body colour, 43 (88%) are green. All species belonging to gonapsis groups G1 and G2 are green. Six species (12%) have a body coloration between grey and yellowish brown. All grey or brown species are in the P4 clade, but in different clusters with mixed body coloration in gonapsis groups G3 and G4.

EGG COLOUR

Mode of egg deposition and egg colour are known from 45 of the 49 taxa treated here. Twenty-eight of those 45 taxa (62%) deposit green eggs, ranging from bright

Figure 4. Gonapophyses of singletons that do not fall within the major molecular clades shown in Figure 1. (A) *Apertochrysa edwardsi*, (B) *Pseudomallada inornatus*, and (C) *Pseudomallada inopinatus*.

yellowish green to dark blue green. Seventeen taxa (38%) deposit white eggs, which can be plain white, beige or greyish, but without any green.

White eggs are distributed across all groups except clade P3 and gonapophysis group G2. All species with a grey or brown body deposit white eggs, but nine taxa with a green body colour also deposit white eggs.

MODE OF EGG DEPOSITION

Eggs are often deposited singly on a pedicel (28 of 45 taxa, 62%), either isolated or in groups or rows. Other species deposit eggs in bundles with loosely or tightly connected pedicels (17 taxa, 38%; e.g. Fig. 2C). Egg bundles occur mainly in the P4 (G3 and G4) cluster. In clusters P1 (*alceste*-group) and P3 (*prasinus*-group), only *P. astur*, *P. formosanus*, and *P. marianus* lay bundled eggs. All of the six species with a grey or brown body colour deposit whitish eggs, five in bundles, one singly.

DISCUSSION

The genus *Pseudomallada*, comprising at least 166 described species, vies with *Leucochrysa* MacLachlan as the largest genus in the Chrysopidae. Almost a third of those are species described from China in the years after 1988 (Dong et al., 2004). Of the remaining 110 species, more than a third were available for study here.

THE FOUR PSEUDOMALLADA GROUPS

Aspöck et al. (2001) considered the species-rich genus *Pseudomallada* (then *Dichochrysa*) in need of revision

and suspected that it was not a monophyletic genus. Now we have evidence that the genus is monophyletic. Additionally, both molecular data and genital morphology support the same four distinct morphological species-groups within the genus. In only three cases, molecular analyses revealed singletons as sister taxa of molecular clades. In two of those, the shape of the gonapophyses leans towards one of the four morphological groups. Thus, there are very few mismatches between molecularly determined clades and morphologically based gonapophysis groups.

In most of the four species-groups, species distributions permit speculation on origins of the groups. The molecularly well-supported P1 clade most likely originated in Asia (*P. astur*, *P. alceste*, *P. sp. 1* and *P. sp. 2*, *P. cognatellus*, *P. parabolus*, *P. formosanus*, *P. ussuriensis*), with two independent range extensions into the Indian Ocean and Africa. *Apertochrysa eurydera* is present on most islands east of Africa and in eastern and northwestern parts of continental Africa. A small clade that includes the widespread species, *P. sjostedti*, also extends to the Mascarene Islands (*P. duplicatus*) and into southern Africa (*P. pulchrinus*).

The easily delineated *prasinus*-group (P3, G2) occurs mainly in Eurasia and northern Africa. Some of the species are difficult to identify reliably, especially the females. This might be the reason for the species names *prasinus* and *abdominalis* occurring several times on the list in Figure 1. We suspect that the *prasinus*-group comprises several undescribed cryptic species. In fact, Aspöck et al. (1980) reported that in large parts of northern Europe, *P. prasinus* and *P. ventralis* form mixed phenotypes. Additionally, the present study shows that except for *P. benedictae*, the species in the *prasinus*-group have similar DNA sequences for the three nuclear genes (*P*-distances are less than 0.009; Table 4). Indeed, sequences for all three genes were nearly the same in Swiss *P. prasinus* and *P. abdominalis*, while Greek *P. prasinus* and Italian *P. abdominalis* had *ATPase* sequences identical to each other. We could not identify cryptic species within the *prasinus*-group. However, *P. marianus* is the only species within the *prasinus*-group to deposit its eggs in bundles, possibly confirming its validity as a separate, cryptic species (Duelli, 1994).

The *flavifrons*-group (part of P4, G3) is mainly African [*P. hamatus*, *P. myassalandicus*, *P. handschini*, *P. chloris*, *P. spissinervis*, *P. sp. gray* from South Africa, *P. luaboensis*, *P. gunvorae*, *P. kibonotoensis*, and *P. baronissus* (recently synonymized, probably prematurely, with *P. kibonotoensis*)]. However, one small, well-supported molecular clade has colonized North America (*P. perfectus*, *P. macleodi*, *P. luctuosus*), while another small clade, which includes the widespread

Table 4. Evolutionary divergences among taxa of *Pseudomallada prasinus*-group (P3 clade) estimated as the number of base substitutions per site averaged over all sequence pairs between the taxa

	<i>P. benedictae</i>	<i>P. zelleri</i>	<i>P. prasinus</i> (Japan)	<i>P. ventralis</i>	<i>P. prasinus</i> (Switzerland)	<i>P. abdominalis</i> (Switzerland)	<i>P. marianus</i>	<i>P. abdominalis</i> (Italy)
<i>P. benedictae</i>	–							
<i>P. zelleri</i>	0.009	–						
<i>P. prasinus</i> (Japan)	0.015	0.009	–					
<i>P. ventralis</i>	0.012	0.007	0.012	–				
<i>P. prasinus</i> (Switzerland)	0.009	0.006	0.008	0.007	–			
<i>P. abdominalis</i> (Switzerland)	0.009	0.006	0.007	0.006	0.001	–		
<i>P. marianus</i>	0.013	0.008	0.012	0.008	0.006	0.005	–	
<i>P. abdominalis</i> (Italy)	0.010	0.005	0.008	0.006	0.003	0.002	0.004	–
<i>P. prasinus</i> (Greece)	0.011	0.007	0.008	0.007	0.006	0.004	0.007	0.003

Uncorrected *P*-distances are shown.

species *P. flavifrons*, extends across Europe (*P. ibericus*, *P. picteti*) and into Asia (*P. flavifrons*).

The *venosus*-group (P5, G4) may have its origin around the Mediterranean Sea [*P. clathratus*, *P. genei*, *P. venosus* (also in western Asia)], but one cluster of species extends into Africa (the widespread *P. nicolai-nus*, the undescribed *P. sp.* Sodere from Ethiopia) and even as far as South Africa (*P. karoensis*, *P. rubicundus*, *P. tactus*).

There are only a few singletons between the molecular clades:

Apertochrysa edwardsi is an Australian species. It clearly belongs to *Pseudomallada* based on molecular data, but it has no tignum. Therefore, it had been placed in the genus *Apertochrysa*, which Brooks & Barnard (1990) reported as having a small, T-shaped gonapsis but no tignum. *Apertochrysa eurydera* similarly lacks a tignum but appears to be a member of the P1 molecular clade. It is clear that at least *A. eurydera*, and probably *A. edwardsi*, should be moved into *Pseudomallada s.s.*, based on molecular data and the shape of the gonapsis. In fact, the gonapsis of *A. edwardsi* is very similar to that of *P. alcestes* and *P. sjostedti*. However, the gonapsis of *A. eurydera*, which is allied on molecular grounds with the *alcestes*-group, instead more closely resembles that of the *flavifrons*-group, or even that of *P. inopinatus*. In any case, our results suggest that the tignum is not definitive for the genus *Pseudomallada*, even though both structures are present in all species of *Pseudomallada s.s.* that we examined. Tsukaguchi's report of no gonapsis in *Apertochrysa* is probably in error, likely caused by accidental substitution of the word gonapsis for tignum on page 18 of his monograph (Tsukaguchi, 1995).

Three species of the genus *Apertochrysa* (*A. albolineatoides* Tsukaguchi, *A. eurydera*, and *A. edwardsi*) are presently scattered across the phylogenetic tree of the Chrysopidae (Fig. 1; Supporting Information, Figs S1 and S2; Haruyama *et al.*, 2008). *Apertochrysa albolineatoides* appears within a clade that includes *Cunctochrysa* Hölzel and *Meleoma* Fitch (Haruyama *et al.*, 2008), while we have shown here the likelihood that *A. eurydera* and *A. edwardsi* are part of *Pseudomallada s.l.* (Fig. 1). These results indicate that a comprehensive systematic revision of *Apertochrysa* is necessary (A. Mochizuki *et al.*, in preparation).

Another singleton is *P. inornatus*, shown in Figure 1 as molecularly distinct from and the sister taxon to P2. The shape of its tiny gonapsis (Fig. 4B) is markedly different from that characterizing any other *Pseudomallada* species. *Pseudomallada inornatus* is known from Europe and the Caucasus.

Pseudomallada inopinatus, a rare endemic highland species on the island of La Réunion east of Madagascar, belongs to the species-rich clade P4, comprising mainly African species. It appears on the tree as an isolated

sister taxon to clade P5. Its gonapsis (Fig. 4C), however, is unlike others in P5. The most similar gonapsis to that of *P. inopinatus* is found in species such as *P. kibonotoensis*, which belong to G4 but are still part of the P4 clade.

ADAPTIVE COLORATION AND OVIPOSITION

Not all 'green lacewings' are green. Some even change their body coloration during the adult's lifetime (Duelli, Johnson, et al., 2014). Variations in body coloration reflect cryptic adaptations to environmental conditions. A well-known characteristic of the genus *Pseudomallada* is the difference in body coloration among closely related species. From Figure 1 it is clear that in two groups (G1 and G2), all species are green, whereas in clade P4 (G3 and G4), some but not all species are grey or brown. These non-green species occur in different sub-clades, interspersed phylogenetically with green species. The conclusion is that green must have been the original body colour in *Pseudomallada*. Grey or brown body coloration, always linked with white or greyish eggs, and often with bundled egg pedicels, is a derived trait, developed independently in dry and hot habitats, where the vegetation is yellow, grey, or brown for most of the period when the adults are active. Even in a bluish-brownish species considered as green here (*P. clathratus*), a yellow mutation occurs in the wild in dry areas (Duelli, 1994). In Europe, brownish-grey species such as *P. venosus*, *P. genei*, or *P. venustus* live only in the driest parts of the continent. Convergent evolution of grey or brown species in different genetic clusters leads to species that look very similar but have completely different shapes of their gonapses.

Not only the colour of the body and eggs is scattered among the phylogenetic clades but also the mode of egg deposition is polyphyletic. Clustered egg pedicels are particularly common in non-green lacewings, but they also occur in some green species in all groups. Depositing eggs singly or in bundles significantly influences the intensity of cannibalism (Duelli & Johnson, 1992) and can be viewed as an adaptive trait (Duelli, 1981). In a habitat with sufficient food for most larvae, depositing eggs singly is a more successful way to produce offspring. With scarce food, isolated tiny larvae soon starve to death, while larvae from bundled eggs have a chance to encounter sibling larvae, which can be attacked and eaten. Laboratory experiments have shown that with scarce food, at least one larva out of a daily bundle of about 20 eggs will survive to the adult stage (Duelli & Johnson, 1992). From the distribution within the clusters in Figure 1 of species with egg bundles, we cannot see an obvious link to particular habitat qualities, other than the observation that bundled eggs are more often deposited in species living in hot

and dry climates. However, several green species living in rather lush habitat (*P. astur*, *P. marianus*, and the North American species) show that there must be other reasons than climate for egg bundles.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Bayesian tree for all chrysopid taxa used in this study. The tree is inferred from a combined analysis of three nuclear genes. The total sequence length of the alignment was 1926 bp, comprising 483 bp of *PepCK*, 525 bp of *wg*, and 918 bp of *ATPase*. Values placed on each node are Bayesian posterior probabilities ≥ 0.80 .

Figure S2. Maximum likelihood tree for all chrysopid taxa used in this study. The tree is inferred from a combined analysis of three nuclear genes. The total sequence length of the alignment was 1926 bp, comprising 483 bp of *PepCK*, 525 bp of *wg*, and 918 bp of *ATPase*. Values placed on each node are ML bootstrap values $\geq 50\%$.