1 **Cryptic lineages of a common alpine mayfly show strong life history divergence**

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9**Running title:** life history divergence of *Baetis alpinus* cryptic lineages

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11ABSTRACT

12Understanding ecological divergence of morphologically similar but genetically distinct
13species – previously considered as a single morphospecies – is of key importance in
14evolutionary ecology and conservation biology. Despite their morphological similarity,
15cryptic species may have evolved distinct adaptations. If such ecological divergence is
16unaccounted for, any predictions about their responses to environmental change and
17biodiversity loss may be biased. We used spatio-temporally replicated field surveys of larval
18cohort structure and population genetic analyses (using nuclear microsatellite markers) to test
19for life history divergence between two cryptic lineages of the alpine mayfly *Baetis alpinus* in
20the Swiss Alps. We found that the more widespread and abundant cryptic lineage represents a
21'generalist' with at least two cohorts per year, whereas the less abundant lineage is restricted to
22higher elevations and represents a 'specialist' with a single cohort per year. Importantly, our
23results indicate partial temporal segregation in reproductive periods between these lineages,
24potentially facilitating local coexistence and reproductive isolation. Taken together, our
25findings emphasize the need for a taxonomic revision: widespread and apparently generalist
26morphospecies can hide cryptic lineages with much narrower ecological niches and
27distribution ranges.

28keywords: *Baetis alpinus*, voltinism, cohort, sympatry, population genetic structure,
29morphospecies

30

31INTRODUCTION

32How biological diversity emerges and evolves is a key question in evolutionary and
33conservation biology (Schluter 2001; Seehausen 2009). An important prerequisite to infer
34evolutionary and ecological processes in natural populations is the appropriate definition of
35evolutionarily distinct units (Crandall *et al.* 2000). One clear challenge in this endeavor is
36posed by cryptic species, which are morphologically similar, yet reproductively isolated and
37genetically distinct (Bickford *et al.* 2007). Cryptic species diversity has become increasingly
38apparent due to the advent of molecular techniques, and suggests that existing criteria and
39practices of species identification are often insufficient (Pfenninger & Schwenk 2007; Jörger
40& Schrödl 2013). As a primary concern, the presence of cryptic species leads to an
41underestimation of biological diversity (Scheffers *et al.* 2012; Adams *et al.* 2014).
42Furthermore, cryptic species can be specialized in their ecology – increasing the risk of
43species loss under environmental change (e.g., Pauls *et al.* 2013; Feckler *et al.* 2014). Beyond
44these important implications for conservation, cryptic species also challenge our
45understanding of population connectivity (Pante *et al.* 2015), speciation, and even ecosystem
46functioning (Seehausen 2009 ; Brodersen & Seehausen 2014).

47If well-recognized species consist of multiple ecologically divergent lineages, any apparently
48wide ecological niche and strong life history plasticity may actually reflect genetically distinct
49specialists (similar to the concept of individual specialization within generalist species,
50Bolnick *et al.* 2003). Furthermore, co-occurrence of morphologically similar species raises
51questions about mechanisms that facilitate divergence and allow species coexistence. For
52instance, stable coexistence of similar species is thought to require some form of ecological
53segregation (i.e., resources, space, or time, Mac Arthur & Levins 1964; Armstrong &
54McGehee 1980; Carothers & Jaksic 1984; Chesson 2000). Such segregation may arise

55through divergence in life history strategies, favoring one or the other competitor under
56different spatially or temporally varying environmental conditions (Qvarnström *et al.* 2005;
57Qvarnström *et al.* 2009).

58At high latitudes or altitudes, seasonally divergent selection may act on the timing of life
59history transitions (Roff 1983; Stearns 1992; Danks 1994; Nylin *et al.* 1995). An
60understanding of how such seasonally divergent selection affects reproduction, population
61dynamics, and genetic divergence on life history traits is crucial in the context of climate
62change (e.g., Kivelä *et al.* 2013). In cryptic species, knowledge about life history divergence
63across environmental mosaics can furthermore provide insight into drivers of adaptive
64divergence and mechanisms of species coexistence.

65Stream invertebrates provide a well-suited study system for testing life history divergence of
66cryptic species. First, as ectotherms, freshwater invertebrates strongly respond to variation in
67temperature and season length. They adjust the timing of their growth period, the number of
68generations produced within a year (i.e., voltinism) and their developmental pathways (e.g.,
69diapause vs. direct development, Masaki 1978; Sweeney 1978). Second, cryptic diversity is
70common in various stream invertebrates (Jackson *et al.* 2014), including baetid mayflies
71(Ephemeroptera) (e.g., Williams *et al.* 2006; Pereira-da-Conceicao *et al.* 2012; Gill *et al.*
722016). Recent work on the alpine mayfly *Baetis alpinus* (Pictet) – a widely distributed and
73phenotypically plastic species (Humpesch 1979; Lavandier 1988; Breitenmoser-Würsten &
74Sartori 1995; Kukula 1997; Erba *et al.* 2003; López-Rodríguez *et al.* 2008) – indicates that
75this species is a composite of at least two genetically distinct lineages (putative cryptic
76species, hereafter termed lineages A and B) (Finn *et al.* 2014; Leys *et al.* 2016). These two
77lineages frequently occur in sympatry in the Swiss Alps (Leys *et al.* 2016). Lineage A is

78widespread, whereas lineage *B* is more abundant at higher elevations – raising the question
79about their ecological divergence.

80In the present study, we used field surveys of larval size distribution in two basins of the
81Swiss Alps, and population genetic analyses to gain insight into life history divergence
82between the two cryptic lineages. First, we used larval cohort structure to determine whether
83the two cryptic lineages differ in voltinism and phenology. We predicted that if the two
84lineages differ in life histories, we should see seasonally variable and distinct larval size
85distribution patterns (possibly indicating different developmental pathways and temporal
86niche partitioning). Second, we studied fine-scale population genetic structure over space and
87time within each cryptic lineage. Specifically, we tested the extent of gene flow across sites
88and between seasonal cohorts (i.e., winter vs. summer cohorts) within the lineages. For the
89latter, low genetic differentiation between winter and summer cohorts would be expected if
90cohorts overlap in their reproductive periods or summer cohorts are produced from the
91previous winter cohorts (or *vice versa*). In contrast, high genetic differentiation between
92winter and summer cohorts would be expected if a given cohort is produced by a previous
93cohort of the same type. Finally, we discuss our findings on life history divergence as a
94putative facilitator of reproductive isolation and local coexistence of cryptic species.

95MATERIAL AND METHODS

96Study species

97*Baetis alpinus* (Pictet) is a common alpine mayfly of the Palearctic. The aquatic egg to larval
98stages are followed by two short-lived aerial stages: the pre-adult (subimago) and the sexually
99mature adult (imago) that only lives for a few days (Studemann *et al.* 1992). The larvae,
100which tolerate a broad range of temperatures, occur in fast-flowing stony streams between

101200 and at least 2600 m above sea level (a.s.l.). The larvae are scrapers and feed primarily on
102diatoms and detritus on rock surfaces (López-Rodríguez *et al.* 2008).

103The life cycle of *B. alpinus* is plastic with regard to emergence timing and voltinism (the
104number of generations per year, Humpesch 1979; Lavandier 1988; Breitenmoser-Würsten &
105Sartori 1995; Kukula 1997; Erba *et al.* 2003; López-Rodríguez *et al.* 2008; Bottová *et al.*
1062013). The number of generations can range from one-half to many within a year (i.e., semi-,
107uni-, bi- to multivoltine). Two cohort types have been described when more than one
108generation per year is produced. So called “winter cohorts” are produced in autumn,
109overwinter as larvae and emerge next spring or early summer. So called “summer cohorts”
110complete egg-to-larval development in summer and emerge in late summer or autumn of the
111same year (Breitenmoser-Würsten & Sartori 1995).

112*Baetis alpinus* is a typical cold-water adapted species (with optimal temperature range of 4-
1138°C) and has strongly temperature-dependent larval and egg development (Humpesch 1979;
114Ward & Stanford 1982; Knispel *et al.* 2006). All cohorts, including winter and summer
115cohorts, overlap and timing of emergence is asynchronous (Breitenmoser-Würsten & Sartori
1161995). Although each adult only lives for a few days, periods of flight (i.e. emergence time)
117can extend over several months (Humpesch 1979). At the adult stage, mating occurs in
118swarms, whereby females fly into male swarms to mate (Brittain 1982). Dispersal occurs
119through adult aerial flight, typically upstream along streams prior to mating, and as
120downstream drift of larvae (Thomas 1975; Lavandier 1982; Schmidt *et al.* 1995).

121Study area and general sampling design

122The study was conducted within two glacier-fed headwater basins of the Swiss Alps, Val
123Roseg and Lötschental, located in the Danube and Rhone drainages, respectively (Figure 1).

Both basins have undergone severe glacial retreat over the last decades, after which *B. alpinus* colonized the newly available area (Bauder *et al.* 2016). These glacierized basins encompass a variety of streams with different dynamics in biogeochemical and physical characteristics (e.g., degree of glacial influence, Tockner *et al.* 1997). In Val Roseg, glacial runoff from the Tschierva and Roseg glaciers is the primary water source of the Roseg river. In Lötschental, the Lonza river is mainly fed by the Lang glacier. In addition, permanently flowing first-order tributaries contribute groundwater and snowmelt to the glacier-fed main channels in both valleys (see Robinson *et al.* 2016, for Val Roseg). Most tributaries have peak flows during spring/summer with increasing groundwater input towards winter (Malard *et al.* 2000). Details of hydrological conditions and geological characterization of each catchment are given in Table S1.

To bracket the environments that *B. alpinus* typically inhabit (Sartori & Landolt 1999), we sampled three spring-fed tributaries and three glacier-fed main-stream sites along a longitudinal gradient downstream from the glacial snout within each basin (Figure 1, Table S2). Sampling took place monthly from April to October/November over two years (2012, 2013). This time period typically covers the period from early larval instars to pre-adult emergence and egg laying (Humpesch 1979). At each site, we collected larvae to i) establish cohort structure based on larval size distributions (all sites; 2012-2013), and ii) identify cryptic lineages and their spatio-temporal distributions (subset of sites, see Figure 1 and Table S2; mostly 2013). One glacial main-stream site in Roseg (RM2) was sampled with higher resolution for genetic analyses (see below). In addition, one tributary site in Roseg (RT2) was investigated fortnightly for the presence of egg clutches from late June to end of October 2013.

147Environmental characterization

148To characterize the environmental variation that developing larvae and eggs experienced, we
149measured several key parameters at each of the 12 study sites: temperature, electrical
150conductivity, periphyton (i.e., the stream biofilm harbouring microbes and detritus on stones)
151and water chemistry. At each tributary site, water temperature was recorded hourly from July
1522012 to October 2013 using a temperature logger (HOBO UTBI-001 TidbiT Temp, Onset
153Computer Corporation, USA). At main-stream sites, the use of temperature loggers was
154prohibited due to channel instability. Here, hourly temperatures were statistically modeled
155based on spot measures and mean hourly data from the nearest hydrological station (2259-
156Rosegbach; 2269-Lonza) provided by the Swiss Federal Office for the Environment (FOEN)
157for 2012 and 2013 (after Ryo *et al.* 2016). Hourly logged data (tributaries) and modeled
158(main-stream sites) temperatures were then used to estimate daily mean temperature for each
159site. To describe the amount of heat accumulated during larval developmental periods, degree
160days (DD) were calculated for each cohort within a lineage at each site. Specifically, DD
161correspond to the sum of daily mean stream temperatures over the presumed growth period of
162a given cohort within each lineage. The actual temperature threshold allowing *B. alpinus*
163larval growth is not known. Therefore, we included only values above 0°C. To avoid bias in
164DD estimates due to different sampling dates, we considered that the larval growth period of a
165given cohort started on the first day of a given month when hatchlings were first observed (at
16601:00) and ended the last day of a given month when last-instar larvae were observed (at
16723:00).

168To quantify resource availability for developing larvae, periphyton biomass was estimated
169monthly using standardized methods (Uehlinger 1991). Periphyton was scraped with a metal
170brush from a known area from the surface of five randomly selected stones at each study site

171on each visit (total N = 360; 5-7 dates in 2013 x 5 stones per site). The brush was rinsed with
172distilled water and the periphyton slurry filtered through pre-ashed glass fiber filters (GF/F,
173Whatmann). In the laboratory, filters were dried at 60°C and weighed, then combusted at
174550°C and reweighed, with the difference in mass after drying and combusting representing
175the ash free dry mass.

176On each sampling date at each site, water electrical conductivity ($\mu\text{S cm}^{-1}$ at 20 °C) was
177measured with a conductivity meter (LF323; WTW, Weilheim, Germany). A water sample
178(0.5 L) was also collected for later chemical analysis in the laboratory (N = 9 dates per site).
179Water samples were stored in polyethylene bottles placed in the dark in a cool box and
180processed within 24h of collection. Conductivity indicates stream ion concentration and
181potential source of water (e.g., glacial melt, groundwater). Other water quality measures taken
182from each water sample included pH, particulate organic carbon (POC), dissolved organic
183carbon (DOC), dissolved organic nitrogen (DN), particulate organic nitrogen (PN),
184phosphate-P (PO_4P), particulate phosphorus (PP), and silicate (H_4SiO_4). These were
185determined in the laboratory according to standard protocols detailed in Tockner *et al.* (1997).

186Life history variation of *B. alpinus*

187To analyze seasonal variation in *B. alpinus* cryptic lineage distributions and abundance, and to
188characterize life cycles, larval samples were collected monthly over two years (Roseg: April
1892012 - October 2013; Lötschental: June 2012 - October 2013). Sampling was done using a
190standard kick-net (250- μm mesh) across all meso-habitats at a given site. To ensure adequate
191sample sizes, sampling was conducted until ca. 150 larvae were collected (if possible) at each
192sampling date and site. In Lötschental main-stream sites, relatively few larvae (7-32
193individuals per site) were found in August 2012 due to an earlier flood. Larvae were
194preserved in 75% ethanol in the field. A subset of larvae were verified in the laboratory

195 following the taxonomic key of Studemann *et al.* (1992), and subsequently preserved in 96%
196 ethanol for later genetic analysis. Given that it was not possible to individually examine each
197 larvae for logistic reasons, and that early-instars are morphologically undistinguishable, the
198 larval size data may include some individuals of the closely related species *B. melanonyx* (see
199 below). This species belongs to the *B. alpinus* species group and is superficially
200 morphologically similar to *B. alpinus* (Bauernfeind & Soldán 2012). In our previous work,
201 *B. melanonyx* was found at low to moderate abundance (2-36%) in Lötschental (Leys *et al.*
202 2016). Although we cannot completely account for bias caused by *B. melanonyx* in the larval
203 size data, we expect this bias to be minimal given the rarity of this species at our study sites
204 (i.e., only 11 out 800 genotyped larvae belonged to *B. melanonyx*; see below).

205 Life cycle analyses were based on temporally repeated measurements of larval size (i.e., total
206 body length) distributions within each site. Larvae were placed laterally on a microscope
207 glass-slide and digital images taken of each specimen at 20-160x magnification (depending on
208 the size of the specimen) using a digital camera mounted on a dissecting microscope (Leica
209 M205C). Total larval body length from the anterior edge of the labrum to the posterior edge
210 of the last abdominal segment was measured from the digital images and fitted as a cubic
211 spline curve using ImageJ software (Schneider *et al.* 2012). A total of 12 641 larvae of the
212 *B. alpinus* species group were measured for size.

213 To account for the effects of sex on size, as well as to gain initial insight into sex ratios at our
214 study sites, the sex of *B. alpinus* larvae was determined for larvae collected in 2013 (N = 2730
215 specimens). An individual was determined as male if it showed a dimorphic structure of the
216 compound eyes, as females lack this trait (Verrier 1956). This sexual character appeared in
217 our dataset for larvae larger than 3.5 mm in size (M. Leys, personal observation).

218Genetic data collection

219DNA was extracted using the HotSHOT method (Montero-Pau *et al.* 2008). To optimize
220representativeness and laboratory effort, two tributary and two main-stream sites per basin
221were included in the genetic analyses. We used this dataset to identify cryptic lineages and to
222quantify within-lineage population genetic structure at 8 of the 12 study sites (see Figure 1).
223To identify the lineage of hatchlings and emergent last-instars, the six smallest and six largest
224individuals within each site and sampling date were genotyped at 10 unlinked and
225polymorphic nuclear microsatellite markers (N = 608 specimens, Leys *et al.* 2016). This
226selection strategy was determined based on an exhaustive genotyping of specimens across all
227size classes at one Roseg main-stream site (RM2) across four sampling dates (N = 192
228specimens). A total of 800 specimens were genotyped. Polymerase chain reaction (PCR)
229amplification, allele-sizing, electrophoresis and genotyping on an ABI 3730xl DNA Analyzer
230(Applied Biosystems) were performed as in Leys *et al.* (2016).

231Statistical analyses

232Environmental variation

233To assess environmental differences among sites and seasonal variation within sites, we first
234ran a principal component analysis (PCA) on eight weakly correlated variables ($r < 0.6$; N =
23577 observations): mean monthly stream temperature, silicate, particulate phosphorus,
236particulate organic nitrogen, particulate organic carbon, periphyton biomass and electrical
237conductivity. We then performed a permutational multivariate analysis of variance
238(PERMANOVA, Anderson 2001) using the environmental dissimilarity matrix based on all
239variables to test for the effects of space and time (basin, stream-type, site and sampling date as
240fixed effects). Significance was assessed using 10 000 permutations in the *vegan* R package
241(R Core Team 2016).

242 Cohort structure at the morphospecies level

243 Cohorts were estimated based on the monthly *B. alpinus* larval sizes, first within the
244 morphospecies and then within each of the two genetic lineages separately. This approach
245 allows us to assess to what extent the variability in voltinism described in the literature (at the
246 morphospecies level) is in fact caused by differences between the two lineages (see
247 discussion). We also took advantage of the large sample sizes of the morphospecies to better
248 allow inference of life-cycle differences between lineages. A cohort was defined to have
249 started when small nymphs (ca. 0.5 to 1-mm size larvae) appeared and to have ended when
250 last-instar individuals appeared (visually distinguishable by wing-pad development and
251 coloration) within a defined time interval.

252 Statistically, each monthly larval size distribution was considered as a mixture of probability
253 density distributions (i.e., the mixture distribution is formed by the combination of individual
254 distributions, the mixture components). We fitted continuous finite mixture models assuming
255 that larval sizes followed a Gaussian distribution using the package *mclust* for R (Fraley &
256 Raftery 2007). For each date and site, models with different numbers of mixture components
257 were compared using the Bayesian information criterion. Each genotyped individual (see
258 below) was then assigned to a particular mixture component using the *predict* function.

259 Once the most likely number of mixture components was determined for each month and site,
260 temporally consecutive mixture components (i.e., assuming that mean larval size increased
261 from hatching to emergence) were assigned to a given cohort (Figure 2, S1). In some cases
262 (RM2: Jul-2012, Jul-2013; RM3: May-2013; RT2: Jul-2012, Jul-AUG 2013; LM3: May-Jul
263 2013; LM5: Jun-2013), the most likely number of mixture components was estimated as one
264 despite the presence of very small (i.e., hatchlings) and large (last instars) individuals – which
265 are unlikely to belong to the same cohort. In these cases, we deduced the most likely number

266of mixture components from the precedent and subsequent month, taking into account
267possible emergence or new recruitment.

268*Cryptic lineage delineation*

269We applied a Discriminant Analysis of Principal Components (DAPC, Jombart *et al.* 2010) to
270assign the subset of individuals (see above) to one of the two cryptic lineages. As a
271preliminary step, microsatellite data from the Roseg and Lötschental basins used in Leys *et al.*
272(2016) were included in the analysis to allow assignment of individuals to the two *B. alpinus*
273lineages (lineages A and B) and to *B. melanonyx*. Eleven individuals (1.4%) were identified as
274*B. melanonyx* and discarded in subsequent analyses. The DAPC analysis also was performed
275for the dataset only including *B. alpinus* individuals (N = 789). For this analysis a total of 50
276principal components (PCs) were retained, accounting for 83.3 % of the total genetic
277variability. This resulted in identical lineage assignments. All analyses were conducted using
278*adegenet* 2.0.1 for R (Jombart 2008).

279*Within-lineage cohorts and spatio-temporal genetic differentiation*

280To test whether the two lineages are randomly distributed within the morphospecies cohorts,
281we calculated the proportion of each lineage within a cohort in a 2 x 2 contingency table for
282each site and performed a Chi-square test of independence (Supplementary Table S3). To gain
283insight into both the approximate mating period (based on the time period when emergence
284occurs), as well as putative temporal isolation in mating time between lineages, adult
285emergence (all sites) and egg laying (Roseg tributary site *RT2*) periods were identified.
286Finally, a factorial ANOVA was used to test tentatively for differences in size between sexes
287and lineages at the onset of emergence. For this, all available last-instar larvae were used (N =
28822, Figure S2).

289The following analyses were performed separately for each cryptic lineage. Genetic
290differentiation among relevant groups (e.g., sites or cohorts) was quantified by the F_{ST} index
291according to Weir & Cockerham (1984). First, we calculated pairwise F_{ST} 's among different
292sites (cohorts being pooled within sites) and, second, between cohorts within a specific site
293using FSTAT (Goudet 1995). To gain insight into the extent of genetic differentiation among
294sites and among cohorts, we estimated variance components attributable to i) individuals
295within cohorts (including only sites and dates with a minimum of eight individuals), ii) among
296cohorts within a site, iii) among sites within stream-type (glacier-fed vs. spring-fed), and iv)
297between stream-type within each basin (see Figure 3), as well as respective hierarchical F -
298statistics using the R package *hierfstat* (Goudet 2005). Statistical significance was assessed
299using 10 000 permutations.

300To visualize the relatedness of the winter and summer cohorts and genetic variation among
301individuals within cohorts, DAPC was performed using cohorts (at a particular site; here only
302sites with a minimum of eight individuals were included) within a given basin as a-priori
303groups (Jombart *et al.* 2010). For lineage A, the analysis was based on 50 PCs in Roseg and
30440 PCs in Lötschental, both accounting for 85.1% of the total genetic variation. For lineage B
305in Roseg (sample size was insufficient in Lötschental), DAPC was based on 25 PCs
306accounting for 87.8% of the total genetic variation.

307Next, we tested for an association between cohort structure and genetic distance between
308individuals (within each site) using a partial Mantel test based on three distance matrices: i)
309individual pairwise genetic distances (Cavalli-Sforza & Edwards 1967), ii) pairwise cohort
310distance (i.e., distance in cohort number for temporally ordered cohorts), and iii) a distance
311matrix indicating whether two individuals belong to one of the three cohort categories:
312summer-summer, winter-winter or winter-summer. The significance of r values was tested

313with a partial Mantel test (10,000 permutations) performed in the package *ncf* for R
314(Bjørnstad 2016).

315Finally, we examined spatial and temporal patterns of genetic variability across cohort types
316(i.e., between summer-summer, winter-winter and summer-winter cohorts) using
317autocorrelation analyses at one main-stream site in Roseg (*RM2* where sample size was
318sufficiently large). We used the kinship coefficient F_{ij} (Loiselle *et al.* 1995) as an
319autocorrelation index and seven 'distance classes' of cohorts (i.e., the distances reflect the
320number of cohorts between temporally ordered cohorts). We calculated confidence intervals
321by permuting cohort number distance (under the null hypothesis of no temporal genetic
322structure) with 10 000 permutations of individuals using SPAGeDI version 1.5 (Hardy &
323Vekemans 2002).

324*Variation related to sex*

325We compared monthly larval size distributions of males and females. Sex-ratios and 95%
326binomial proportion confidence intervals were calculated i) at the morphospecies level for
327each sampling date at a particular site (all sites), and ii) at the lineage level within each cohort
328at the Roseg main-stream site *RM2*. We tested whether sex-ratios deviated from 1:1 within
329these different levels using binomial tests using the function *binom.test* from the R library
330*stats* (R Core Team 2016).

331RESULTS

332Environmental variation

333Ordination of environmental parameters by PCA revealed strong spatial and seasonal
334variation (Figure S3). PERMANOVA on the environmental dissimilarity matrix indicated

335significant effects of stream-type ($R^2 = 0.17$, $F_{1,59} = 24.20$), site ($R^2 = 0.21$, $F_{9,59} = 3.34$), basin
336($R^2 = 0.04$, $F_{1,59} = 5.40$) and season (month: $R^2 = 0.17$, $F_{6,59} = 4.04$; all $P < 0.001$).

337The main-stream sites (ranging from 0.0 to 8.8°C, mean $\pm$ sd: $2.3 \pm 2.0^\circ\text{C}$) were typically
338colder than the tributaries (ranging from -0.8 to 15.4°C, mean $\pm$ sd: $4.4 \pm 3.6^\circ\text{C}$; Figure S4),
339reflecting a glacial influence and larger volume of water. An exception were Roseg main-
340stream sites *RM2* and *RM3*, which were relatively warmer because they are located below a
341lake outlet (Figures 1, S4, Finn *et al.* 2010). Moreover, daily mean temperatures increased
342with distance from the glacier in the main-stream sites (Figure S4). Tributaries differed in
343mean and variation of temperature, likely due to groundwater inputs and coarse-scale spatial
344attributes (e.g., north vs. south-facing slope).

345Mean periphyton biomass increased in autumn (i.e., when glacier run-off decreases in glacial
346streams (Uehlinger *et al.* 2010), and differed also among sites (Figure S5a). The highest
347periphyton biomass was measured at *RT3* in Roseg and *LT4* in Lötschental, indicating higher
348resource availability for developing larvae in these tributaries (Figure S5b).

349Life history variation

350Cohort structure at the morphospecies level

351Larvae of *B. alpinus* were abundant at nearly all sites and sampling dates (the glacial *RM1* site
352in Roseg, however, had abundant early-instar larvae only in early spring; Figures S1, S6).
353Last-instar larvae were found from June to October, suggesting that *B. alpinus* reproduces
354from early summer to autumn at our study sites (Figure S6). This was further supported by
355egg clutches being found from mid-June to late October (data on Roseg tributary *RT2*).

Cohort delineation analysis at the morphospecies level indicated a multivoltine life cycle, with three to four overlapping cohorts (*sensu* Clifford 1982, Figure 2). Specifically, the morphospecies appeared to consist of both winter (i.e., overwintering as hatched larvae with winter/spring larval development and emergence in early summer) and summer (i.e., embryonic and larval development during spring and summer, and emergence in summer and autumn) cohorts (Figures 2, S1 and S6).

Based on larval size distributions, the earliest winter cohort within a given site (henceforth named *a*) hatched in September, with subsequent overwintering as early larval instars, and emergence taking place in late-spring to early summer (e.g., cohort *a2* in Roseg glacial stream-site RM2-2012 and 2013; Figure 2). A second winter cohort (henceforth named *b*) was indicated by the presence of early instars in October-November, concomitant with the presence of larger nymphs and last-instar larvae in late July (e.g., cohort *b2* in Roseg glacial stream-site RM2-2012 and 2013). These two apparent winter cohorts (*a* and *b*) were followed by one or two (depending on site and year) fast developing summer cohorts (*c* and *d*; Figure 2). Development time of the winter cohorts ranged from 10 to 11 months and that of summer cohorts from two to three months (e.g., *c1* in Roseg glacial stream-site RM2-2012, *d1* in RM2-2012). Based on the appearance of last-instar larvae, emergence phenology varied between years and among sites (Figure S6). Monthly larval size distribution for each sex, as well as sex-ratios, suggested that the inferred cohort structure is not strongly confounded by size differences of males and females (Figures S7, S8).

Novel insight from genotyping: cryptic lineage life history divergence

A total of 789 *B. alpinus* individuals were genotyped. Of these, the DAPC unambiguously assigned 642 individuals to lineage A and 147 individuals to lineage B (Figure 4a). The lineages were strongly genetically distinct ($F_{ST} = 0.203$). Both lineages occurred at most sites

380(with the exception of main stream-site *RM3* in Roseg where no lineage *B* individuals were
381found; Figure 4b). Lineage *A* was more abundant than lineage *B* at all sites, with an increasing
382prevalence of lineage *B* at higher elevations (from 0 at lowest site to 26.8% at highest site;
383averaged across sampling dates, Table S3 and Figure 4b).

384The relative frequencies of lineage *A* and *B* individuals differed significantly among the
385morphospecies-based cohorts (Pearson Chi-square test of independence: all $P < 0.0001$ except
386tributary *RT4* in Roseg and main- stream site *LM5* in Lötschental where very few lineage *B*
387individuals were observed; see Table S3), suggesting that the lineages differ in life histories.
388Subsequent lineage-specific cohort analyses support this view. First, lineage *A* appears to
389have a bivoltine (tributaries *LT2*, *LT4* in Lötschental, *RT2* in Roseg) to multivoltine life cycle
390(e.g., main stream-site *RM3* in Roseg), including one to several summer and winter cohorts
391(Figure 4). For the winter cohorts, appearance of small nymphs started typically in September
392- October and last-instar larvae were observed in June - July. The larval period of lineage *A*
393winter cohort spanned 10 to 11 months. For the summer cohort of lineage *A*, hatchlings were
394observed in August and last-instar larvae were observed in September - October, indicating a
395larval period of two-three months. In contrast, lineage *B* appears to be consistently univoltine,
396with a single summer cohort: larval development began in late May, June or early July,
397followed by a growth period of ca. three to five months and emergence in July to September
398(Figure 4b). Based on the appearance of last-instars, emergence occurred from July to October
399in lineage *A* and in August in lineage *B* (Figure 4). This result indicates some temporal
400differentiation, but also temporal overlap in mating times between the lineages.

401Based on the association between the larval development period of a given cohort (within
402each lineage) and thermal conditions during a given period, cohorts of each lineage
403experienced different thermal conditions (Figure 4, Table S4). However, the patterns were

404 somewhat inconsistent between the basins. In Lötschental, DDs of larval developmental
405 periods appeared, on average, higher for lineage A winter cohorts and for lineage B than for
406 lineage A summer cohorts. In Roseg, DDs of lineage A winter cohorts were equivalent or
407 lower than for summer cohorts (Table S4).

408 *Within-lineage population genetic differentiation*

409 The hierarchical analysis of population genetic differentiation within lineage A showed that a
410 significant amount of genetic variance was explained by cohort (within site) in both basins.
411 Genetic variance explained by stream-type was of similar magnitude, but not significant (both
412 $P = 0.16$, Table 1 and Figure 3; possibly due to low statistical power). Pairwise F_{ST} s indicated
413 subtle genetic differentiation between sites and between cohorts (Figure S9). Furthermore,
414 DAPC analyses, using cohorts within each lineage as prior groups, indicated that winter and
415 summer cohorts were moderately genetically differentiated (Figure 5). These results were
416 consistent with both Partial mantel tests and temporal autocorrelation analyses: pairs of
417 individuals where one belonged to a summer and the other to a winter cohort were genetically
418 more dissimilar than pairs where both individuals belonged to summer cohorts or winter
419 cohorts, respectively, even when accounting for possible temporal effects (partial Mantel
420 tests, all $P < 0.01$ except for Roseg main-stream site *RM3* and tributary *RT4*; Table 2 and
421 Figure S10).

422 DISCUSSION

423 Life history trait divergence in morphologically similar but genetically distinct cryptic
424 lineages can provide insight into adaptation, mechanisms of species coexistence, as well as
425 population dynamics. Our study on *B. alpinus* indicates clear divergence of two co-occurring
426 cryptic lineages in life cycles and spatio-temporal dynamics. Specifically, we found that
427 lineage A is relatively more abundant locally and has a bi- to multivoltine life cycle, whereas

lineage *B* has a consistently univoltine life cycle. In a previous study, we further showed that lineage *A* is more widely distributed across the Swiss alps and harbors more neutral genetic diversity than lineage *B* (Leys *et al.* 2016). Taken together, these findings support the view that lineage *A* represents a generalist lineage, whereas lineage *B* likely is more specialized to high mountain conditions. We next discuss the implications of such life history divergence for the evolutionary ecology of cryptic lineages in general and for *B. alpinus* specifically.

Contrasting inference from morphospecies vs. cryptic lineage life-cycles

The accuracy of biodiversity assessments and predictions of responses to climate change strongly depends on a robust analysis of species boundaries and differences in ecology (Pauls *et al.* 2013). This point is clearly illustrated in our comparisons of life cycles based on morphospecies vs. cryptic lineage identities. At the morphospecies level, analysis of larval cohort structure in *B. alpinus* indicated the presence of two or more generations at most study sites. However, the two cryptic lineages clearly showed different life history strategies. First, lineage *A* had a bivoltine to multivoltine life cycle that consisted of winter cohorts (overwintering as larvae) and summer cohorts (completing larval development from hatching to emergence in summer). In contrast, lineage *B* was consistently univoltine with one fast growing summer cohort at all sites (larval development completed in 3-5 months; Figure 4).

The lineage *A* winter and summer cohorts differed in the length of the larval period (10-11 months in winter cohorts vs. 2-3 months in summer cohorts) and thereby winter and summer cohorts experience different larval growth conditions. The winter cohort hatched in September-October and emerged first in June-July of the following year. Hence, the larvae presumably faced very low temperatures slowing down (or possibly even arresting) development until temperatures increase in spring. In contrast, larvae of the summer cohorts started developing in July-August and emerged in September-October. These larvae therefore

452likely faced favorable growth conditions resulting in faster growth and shorter developmental
453periods.

454Interestingly, lineage *B* showed yet another life history pattern. The univoltine life cycle of
455this lineage consisted of summer cohorts, which – in contrast to lineage *A* – appeared to
456overwinter as embryos. Embryonic diapause is indicated by emergence (and also egg laying at
457Roseg tributary, *RT2*) taking place in summer/autumn, but hatchlings not being detected
458before the following spring (Figure 4). This observation is intriguing. Although the life cycle
459of *B. alpinus* has been extensively studied over decades (e.g., Humpesch 1979; Lavandier
4601988; Breitenmoser-Würsten & Sartori 1995; Kukula 1997; Erba *et al.* 2003; Knispel *et al.*
4612006; López-Rodríguez *et al.* 2008; Bottová *et al.* 2013), our study provides the first
462indication of embryonic diapause in this species.

463To what extent these differences in life-cycles between the two lineages in the wild reflect
464phenotypic plasticity and/or genetically determined differences in developmental pathways is
465thus far unclear. Given that ectotherms (including *B. alpinus*) strongly respond to variation in
466thermal conditions during development, environmentally induced phenotypic plasticity is
467certainly likely (Angilletta 2009). However, genetic divergence in developmental pathways–
468in response to divergent natural selection via temperature and seasonal time constraints – is
469also possible. Such divergence has been shown in damselflies (Sniegula *et al.* 2016),
470*Drosophila* (Barker *et al.* 2011), frogs (Muir *et al.* 2014) and other taxa (reviewed in Keller *et al.*
4712013). Clearly, further studies on determinants of life-cycle variation in this system are
472needed. Here experiments under controlled laboratory conditions, concomitant to genomic
473tools to establish the genetic basis of trait divergence, would be particularly useful.

474 *Some caveats*

475 Our study comes with some caveats that need to be addressed in future studies. First, the
476 presence of young and intermediate-instar larvae continuously throughout the year did not
477 always allow us to unambiguously determine the beginning and end of a given generation
478 (Figures 2, S1, S6). However, two approaches helped to increase accuracy of cohort
479 assignments. Monthly larval size distributions combined with statistical inferences (maximum
480 likelihood estimation of Gaussian mixture models), allowed us to determine cohorts more
481 objectively than the often-used visual inspections of size frequency histograms (e.g., Erba *et*
482 *al.* 2003; López-Rodríguez *et al.* 2008). Likewise, monitoring the presence/absence of eggs at
483 one tributary (RT2) allowed us to link the temporal presence of eggs to the appearance of
484 hatchlings.

485 A second potential source of error for cohort inferences comes from using pooled data from
486 males and females (it was not possible to reliably sex all larval stages). This could introduce
487 bias to life-cycle estimates if males and females differ in larval growth patterns (i.e., part of
488 the variation may be due to sex ratio differences over time or space). Such sex-specific
489 differences have been shown in *B. rhodani*, a mayfly genetically related to *B. alpinus*:
490 *B. rhodani* females are larger than males at emergence because they have more instars and
491 emerge later than males (Elliott 2013). Our data tentatively indicate that sex ratios may differ
492 across sites (possibly between lineages) and that the sexes differ in size at emergence (males
493 being smaller than females, Figure S2). However, life history divergence of lineages was
494 consistently detected at multiple sites, suggesting that effects of sexual dimorphism on cohort
495 structure were minor. Given the small sample sizes further studies on sex-specific variation in
496 life-cycles are warranted however.

497 **Alternative life history strategies and lineage coexistence**

498 Seasonal environments can impose strong selection on reproduction and organismal
499 development due to environmental fluctuations and time constraints (Roff 2002; Kivelä *et al.*
500 2013). The adaptive value of the life-cycle variation seen here need yet to be directly tested,
501 but several adaptive explanations are likely. First, direct development (as apparent in lineage
502 A) can allow more generations per year than a life-cycle with embryonic diapause (as
503 presumably is the case in lineage B). On the other hand, embryonic diapause likely represents
504 an adaptive overwintering strategy that prevents exposure of temperature-sensitive larval
505 stages to unfavorable conditions (e.g., Tauber & Tauber 1981). Furthermore, the presence of
506 multiple overlapping cohorts and variation in emergence time (as in lineage A) may be
507 adaptive in unpredictable environments. In alpine environments, unpredictable climate
508 variation (Scherrer *et al.* 2004) may favor a bet-hedging strategy, whereby the distribution of
509 reproduction over the entire season may reduce the probability that entire generations are
510 wiped out by catastrophic events (Kiss & Samu 2005; Wilbur & Rudolf 2006). Finally,
511 competition for resources may favor a mixture of larval phenotypes and induce density-
512 dependent processes that favor different hatching and emergence periods (Iwasa *et al.* 1994;
513 Elliott 2013).

514 The observed spatio-temporal differences in larval cohort structure and egg laying periods
515 indicate some seasonal partitioning between the two cryptic lineages (Figure 4). Such
516 temporal divergence may reflect past divergent selection in geographic isolation and
517 subsequent secondary contact, or evolutionary divergence *in situ*. Irrespective of the
518 evolutionary history, such seasonal segregation may favor the coexistence of these closely
519 related and morphologically similar lineages by minimizing the temporal overlap in resource

use and competition (Armstrong & McGehee 1976; Armstrong & McGehee 1980; Chesson 1985; Chesson 2000). These hypotheses need to be explicitly tested, however.

Within-lineage population genetic differentiation

We found subtle but significant neutral genetic divergence across our study sites, indicating some spatial limits to gene flow, but no clear evidence for habitat isolation within lineages. Given the highly dynamic nature of alpine basins (possibly favouring metapopulation dynamics), such moderate spatial isolation within basin is not surprising. More interestingly, we found moderate and significant genetic structure between seasonal cohorts within lineage A. Specifically, winter cohorts were genetically more related to other winter cohorts than to the subsequent summer cohorts (which would be nearest in terms of absolute time). This suggests that a given winter cohort produces the next winter cohort and that individuals from different summer/winter cohorts do not represent a panmictic population. It is also possible, however, that winter cohorts partly produce summer cohorts, but that the cohort types are genetically distinct due to, for instance, founder effects (Lenfant & Planes 2002). Interestingly, Landa (1969) found differences in larval morphology (i.e., in the middle caudal filament) between winter and summer cohorts of bivoltine populations in *B. alpinus*. Assuming this variation does not reflect unknown cryptic species (an option we cannot exclude, although we found no evidence for such differences between lineage A and B, Leys *et al.* 2016), this finding would further support ecological and genetic differentiation between cohort types. Overall, our results suggest complex spatial and temporal patterns of gene flow, likely resulting from within basin interactions between life history variation, adult dispersal and local bottlenecks (e.g., due to hydroperiod mediated variation in oviposition site availability, Peckarsky *et al.* 2000; Caudill 2003).

543General implications and outlook

544Here we provide novel evidence of life-cycle divergence between two cryptic lineages of a
545widely distributed alpine mayfly. When undetected, such ecological divergence between
546cryptic lineages can confound estimates of the spatial distribution and ecological niche of a
547species (Leys *et al.* 2016). Specifically, unreliable estimates of environmental requirements,
548life histories and reproductive characteristics of organisms can have significant repercussions
549for many ecological and evolutionary research fields, as well as estimates of biodiversity (e.g.,
550Feckler *et al.* 2014; Pante *et al.* 2015). Our results clearly support the need for accurate
551delimitation of evolutionary units (Crandall *et al.* 2000; Brodersen & Seehausen 2014). In
552particular, instead of a highly variable and phenotypically plastic species with a wide
553ecological niche (as previously has been repeatedly suggested), *B. alpinus* is a mix of
554apparent generalists (here lineage A) and specialists (here lineage B) that are reproductively
555isolated.

556Yet several key questions are left open for our study system. First, what is the adaptive value
557of these generalist and specialist strategies as well as their responses to climate change?
558Ultimately these are determined both by fitness benefits and trade-offs, and extent of
559phenotypic plasticity *versus* heritable genetic variation (Merilä & Hendry 2014), necessitating
560explicit experimental tests in the future. Second, what are the mechanisms of reproductive
561isolation between these divergent lineages? The lineages showed partial temporal segregation
562in reproductive periods, but additional quantitative field data on key reproductive parameters
563(e.g., emergence times, swarming sites, sex ratios of emerging adults) would be particularly
564valuable for estimating putative overlap (or lack thereof) of mating times and sites between
565the lineages. Finally, what are the processes that produced this life history divergence and to
566what extent does this divergence influences co-existence of these morphologically similar

567lineages? On one hand, this divergence may have occurred in response to divergent natural
568selection unrelated to resource use, but currently facilitate spatial coexistence due to reduced
569inter-specific competition. Alternatively, resource competition may have directly facilitated
570ecological trait divergence and reproductive isolation (i.e., ecological character displacement,
571Pfennig & Pfennig 2009; Stuart & Losos 2013). Further research is needed to infer whether
572the observed divergence was a result of past independent adaptation to different niches and/or
573a result of sympatric evolution *in situ*. Given likely different historical distribution ranges of
574these lineages and secondary contact upon range expansion, past isolation is certainly possible
575(Leys *et al.* 2016). Our study highlights the value of temporally replicated phenotypic and
576genetic surveys of cryptic lineages in the wild: integrating such data can aid in assessing life
577history variation of cryptic species and is critical for understanding their evolutionary past and
578their future responses to environmental change.

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814DATA ACCESSIBILITY

815Nuclear microsatellite data, larval size measurements and environmental data (temperature,
816periphyton and water chemistry) are available from the Dryad Digital Repository:
817<http://dx.doi.org/10.5061/dryad.kt364>

818AUTHOR CONTRIBUTIONS

819ML, IK, CTR and KR conceived and designed the study. ML and CTR performed field
820sampling. ML carried out part of the laboratory work, analyzed the data and prepared the first
821manuscript draft and all authors contributed substantially to the writing of the manuscript. All
822authors read and approved the final manuscript.

823

824 TABLES

825 **Table 1.** Hierarchical variance partitioning and F -statistics of population genetic
 826 differentiation within lineage *A* (there was insufficient data for lineage *B*). Analyses were
 827 performed separately within the Lötschental and Roseg basins. Significant values ($P < 0.05$)
 828 are shown in bold.

Variance components

	Stream	Site	Cohort	Individual	Error
Lötschental	0.160	0.020	0.100	0.650	6.900
Roseg	0.110	0.020	0.110	0.680	6.990

Hierarchical F -statistics

	$F_{\text{STREAM-TOTAL}}$	$F_{\text{SITE-STREAM}}$	$F_{\text{COHORT-SITE}}$	$F_{\text{IND-COHORT}}$
Lötschental	0.020	0.003	0.013	0.086
Roseg	0.014	0.002	0.014	0.088

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Table 2. Correlation coefficients of Partial Mantel tests i) between individual pairwise genetic distances (G) (Cavalli & Sforza 1969) and temporal distances (T) (i.e. Matrix pair G x T), when cohort type (C) was controlled for, and ii) between genetic distances and cohort type (i.e. Matrix pair G x C), when temporal distance was controlled for. The temporal distance matrix is expressed as cohort number for temporally ordered cohorts within each study site (see Figure 2 for details). The cohort type matrix indicates which of the three cohort categories a pair of individuals belongs to (i.e. summer-summer, winter-winter or winter-summer cohort pairs). Significant values ($P < 0.05$) are indicated in bold.

Site	Matrix pair tested	Matrix controlled	r	P
<i>LM3</i>	G x T	C	-0.018	0.271
<i>LM3</i>	G x C	T	0.226	0.002
<i>LM5</i>	G x T	C	-0.051	0.064
<i>LM5</i>	G x C	T	0.156	0.005
<i>LT2</i>	G x T	C	0.059	0.098
<i>LT2</i>	G x C	T	0.173	0.012
<i>LT4</i>	G x T	C	0.180	0.001
<i>LT4</i>	G x C	T	0.318	0.001
<i>RM2</i>	G x T	C	0.028	0.134
<i>RM2</i>	G x C	T	0.189	0.000
<i>RM3</i>	G x T	C	0.042	0.150
<i>RM3</i>	G x C	T	0.006	0.408
<i>RT2</i>	G x T	C	-0.001	0.495
<i>RT2</i>	G x C	T	0.242	0.000
<i>RT4</i>	G x T	C	0.217	0.000
<i>RT4</i>	G x C	T	-0.004	0.487

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FIGURE LEGENDS

Figure 1. (a) General location of study areas within Europe and (b) the two glacierized study basins (indicated by boxes) in Switzerland. (c) Sampling sites are visualized at the basin scale and labeled as in Leys *et al.* (2016). Main glacier-fed channel sites and tributaries are indicated by white and dark grey, respectively. Dotted arrows show river flow direction. Sites for which genetic data were available are marked with an asterisk.

Figure 2. *B. alpinus* cohorts determined by Gaussian mixture models of monthly larval size density distributions (only for sites for which genetic data were available. Size based cohorts for all remaining sites are given in Figure S1). Cohort identification was based on models of mixture components (see material and methods for details). A given cohort type (winter cohorts *a* and *b* and summer cohorts *c* and *d*) is depicted by the same color within and between seasonal cycles. The numbers (1, 2, 3) distinguish cohorts across two seasonal cycles (e.g., *a1* and *a2* represent the first winter cohorts in 2012 and 2013, and *b1* and *b2* the second winter cohorts, respectively).

Figure 3. Scheme of the hierarchical levels included in the hierarchical analysis of population genetic differentiation within a lineage (see Table 1 for statistical analyses).

Figure 4. Genetic assignment of 789 individuals to *B. alpinus* cryptic lineages based on 10 microsatellite markers in two glacierized basins of the Swiss Alps. (a) Individual assignment to A (blue) and B lineages (green) using DAPC. The x-axis shows the values of the first discriminant function for each individual (vertical bars) and the y-axis indicates the smoothed density of observations. (b) Violin plots of spatio-temporal distribution of *B. alpinus* lineages A and B in Lötschental and Roseg in 2012 (upper panels) and 2013 (lower panels). The few *B. melanonyx* individuals identified based on genetic data are also indicated. Each colored symbol corresponds to an individual (Blue: lineage A; Green: lineage B; Orange: *B. melanonyx*). Each violin plot represents the larval size distribution at the morphospecies level. Larval body length (mm) is indicated on the y-axis. Dots and triangles indicate early to intermediate larval stages and last-instars, respectively. For Roseg tributary RT2 (in 2013), the presence of egg clutches is indicated for each lineage by filled squares above the panel.

Figure 5. Genetic affinities between cohorts within each *B. alpinus* lineage visualized by discriminant analysis of principal components (DAPC) on 10 microsatellite markers in (a) lineage A in Lötschental, (b) lineage A in Roseg, and (c) lineage B in Roseg. Scatterplots show the first two principal components of the DAPC. Eigenvalues corresponding to the represented components are filled in black in the right insert. The number of principal components retained and their cumulative variance explained are highlighted in black in the left-insert. The cohorts (*a*, *b*, *c*, *d*) within each annual cycle are depicted by colors (as in Figure 2).

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877Figure 1.

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b.

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884 **Figure 3.**

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887Figure 4.

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b.

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